**Transferrin cleavage during acute inflammation in the goldfish, *Carassius auratus***

by

Michael Trites

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

Master of Science

in
Physiology, Cell, and Developmental Biology

Department of Biological Sciences
University of Alberta

# Abstract

Transferrin is an evolutionary conserved protein that in addition to having a critical role in iron transport also has been shown to have a crucial role in host defence. Transferrin has been shown to sequester iron from invading pathogens, act directly against microbial pathogens, and is an evolutionary conserved acute phase protein. Recently, cleaved transferrin products have been shown to activate both teleost and murine macrophages *in vitro*. The objectives of my thesis were to characterize the presence and regulation of cleaved transferrin products to acute inflammation. I used an *in vivo* model of self-resolving inflammation in goldfish, coupled with analysis of leucocyte anti-microbial analysis responses. Specifically: gene expression, leucocyte influx, respiratory burst, and nitric oxide production. I also used protein analysis to evaluate the contributions of cleaved transferrin to acute inflammation. I show, for the first time, that cleaved transferrin products are produced *in vivo* during an acute inflammatory response. I initially investigated the ability of cleaved transferrin fragments to serve as a broader marker of the acute inflammatory response compared to short-lived and low concentration cytokines. Cleaved transferrin fragments were produced during pathogen induced, but not sterile, inflammation. However there was a large degree of heterogeneity in banding patterns within transferrin cleavage products detected by Western blot, and there are likely a multitude of cleavage products generated *in vivo* that were undetected with the primary antibody used. I then investigated the potential contributions of transferrin cleavage products during acute inflammation *in vivo*. Macrophages, but not neutrophils, potentially contribute to production of transferrin through inducible expression of transferrin during inflammation. Pro-inflammatory neutrophils, in contrast,

displayed a preferential ability to enzymatically digest transferrin; that was reduced in macrophages and late-phase pro-resolving neutrophils. This study adds to a growing body or work highlighting the role of transferrin as an immune regulator during acute inflammation. Given the significant conservation of this and related molecules, these findings have potentially broad implications for host defences and inflammation control across evolution. Overall the data presented suggests that cleaved transferrin products may play a role during activation events of macrophages *in vivo* and this mechanism is likely conserved throughout evolution.

# Preface

This thesis is an original work by Michael Trites. 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.

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

# Acknowledgements

There are many people who have helped me along the course of my Masters program. I would first like to thank the Natural Sciences and Engineering Research Council and the University of Alberta for funding these projects.

I would like to thank all of the animal care staff. Specifically Jesse Edgington, Clarence Gerla, Simmone Kerswell, and all of the other aquatics staff for all of their help in fish care. I would also like to thank the graduate student support staff of Biological Sciences, Chesceri Gafuik and Melany Rattray who have helped me navigate the, often confusing, path of administrative details.

I would like to thank the current and past members of the "Barreda Lab" with whom I have had the pleasure of working with over the past three years. Whether it be advice in setting up experiments, or bouncing ideas off of I could always count on assistance when it was required. Along with the rest of the third floor centre wing gang, you made working in the lab a pleasure.

Thank you to my supervisory committee, Dr. John Chang and Dr. James Stafford for their analysis and feedback on my research. Thank you to Dr. Basil Hubbard for his role as the arm-length's examiner and to Dr. Martin Sryako for his role as the chair.

My final thank you goes to my supervisor, and mentor, Dr. Dan Barreda. His mentorship and guidance over the past three years has been instrumental in helping me design experiments, interpret results, and more importantly grow as a scientist. I have been incredibly fortunate to be able to work in a lab with the resources available and with such a passionate mentor.

# Table of Contents

# List of Tables

# List of Figures

# List of abbreviations

| | |
|---|---|
| **cDNA** | Complementary DNA |
| **CFU** | Colony forming units |
| **COX** | Cyclooxygenase |
| **DAF-FM** | 4-amino-5-methylamino-2',7'-difluorofluorescein |
| **DAMPs** | Damage-associated molecular patterns |
| **DHR** | Dihydrorhodamine |
| **DMEM** | Dulbecco's modified Eagle media |
| **DNA** | Deoxyribonucleic acid |
| **eNOS** | Endothelial nitric oxide synthase |
| **FACS** | Florescence activated cell sorter |
| **$G_1$ phase** | Gap 1 phase |
| **HBSS** | Hank's balanced salt solution |
| **HRP** | Horse-radish peroxidase |
| **HSP** | Heat shock protein |
| **IFN** | Interferon |
| **IL** | Interleukin |
| **iNOS** | Inducible nitric oxide synthase |
| **kDa** | Kilo-Dalton |
| **LPS** | Lipopolysaccharide |
| **M-phase** | Mitosis phase |
| **MGFL-15** | Modified goldfish Leibovitz-15 |
| **mRNA** | Messenger ribonucleic acid |
| **NADPH** | Nicotinamide adenine dinucleotide phosphate |
| **NETs** | Neutrophil extracellular traps |
| **NK** | Natural killer |
| **NLRs** | Nod-like receptors |
| **NO** | Nitric oxide |
| **nNOS** | Neuronal nitric oxide synthase |
| **NSAID** | Non-steroidal anti-inflammatory drugs |
| **PAMPs** | Pathogen-associated molecular patterns |
| **PBS** | Phosphate buffer saline |

| | |
|---|---|
| **PCR** | Polymerase chain reaction |
| **$PGE_2$** | Prostaglandin $E_2$ |
| **PKM** | Primary kidney macrophages |
| **PMA** | Phorbol myristate acetate |
| **Poly(I:C)** | Polyinosinic:polycytidylic acid |
| **PRR** | Pattern recognition receptor |
| **RNA** | Ribonucleic acid |
| **RNI** | Reactive nitrogen intermediates |
| **ROI** | Reactive oxygen intermediates |
| **ROS** | Reactive oxygen species |
| **RT-PCR** | Reverse transcriptase polymerase chain reaction |
| **SDS** | Sodium dodecyl sulfate |
| **TBS-T** | Tris buffer saline - Tween |
| **TCR** | T-cell receptor |
| **TGF** | Transforming growth factor |
| **TLRs** | Toll-like receptors |
| **TMS** | Tricane methane sulfonate |
| **TNF** | Tumour necrosis factor |
| **TSA** | Tryptic soy agar |

# Chapter 1: Introduction and Literature Review

## 1.1 Introduction

Transferrins are molecules that are essential for survival due to their pivotal roles in iron metabolism and host defence. Transferrin and transferrin-like molecules are highly conserved with the iron-binding properties of transferrin-like molecules being demonstrated in all organisms, dating back to marine-algae. Transferrins are also crucial for effective host defence in several evolutionary lineages, with current evidence showing that transferrin has immunoregulatory functions in animals from arthropods to humans. Transferrins have been shown to restrict iron from pathogens, preventing growth and proliferation. Transferrins have evolved several direct roles in host defence both by regulating host immune responses and acting directly on pathogens. However pathogens also participate in this evolutionary arms race, developing several strategies to circumvent host manipulation of transferrin for their own benefit.

The innate immune system is essential to providing effective defence against invading microorganisms. Acute inflammation is one of the earliest mechanisms hosts use to combat infiltrating pathogens. Acute inflammation involves a rapid response to pathogenic insult, or tissue damage, that is characterized by highly conserved phagocyte-mediated responses. The initiation, effector, and resolution phases of these phagocytes tightly regulate acute inflammation by the multi-orchestrated release of soluble mediators to both pathogenic and homeostatic signals. These initial responses are not only critical to effective mitigation of pathogen infiltration and return to homeostasis but also critical to effective development of an acquired, memory response. These responses have been well characterized in mammalian systems. Teleost (bony) fish have recently been shown to

posses many similar mechanisms to mammalian immune responses, and are one of the earliest evolutionary clades that possess both innate and classical adaptive immune responses. Teleosts serve as an excellent comparative model for investigating primitive mechanisms for the control of acute inflammation. In my thesis I investigate the potential role of transferrin cleavage products as a primitive mechanism for its contributions to regulation of acute inflammation *in vivo*.

## 1.2 Objectives and hypothesis

The objectives of this thesis are to investigate the ability of cleaved transferrin products to serve as a broad marker of induction phase of acute inflammation. Current markers of the induction phase of acute inflammation centre on cytokines (TNF-α, IL-1β, IFN-γ), which are synthesized and utilized rapidly and in small quantities. Given that transferrin plays an integral role of host defence, conserved throughout evolution, and present in high concentrations, **I propose that cleaved transferrin products could serve as a good comparative marker of the early phases in acute inflammation**.

I also plan to investigate the potential role of cleaved transferrin products during acute inflammation. I will use a self-resolving model of inflammation in goldfish, *Carassius auratus,* to investigate the roles of transferrin at the site of inflammation *in vivo*. To examine these characteristics I will use a combination of gene expression- and protein analysis, coupled with evaluating well-characterized leucocyte mediated anti-microbial responses**. I hypothesize that transferrin cleavage products are generated early during acute inflammation and play a role in initial activation cascades of macrophages in the induction of acute inflammation**.

## 1.3 Outline of thesis

This thesis is comprised of 5 chapters. The first chapter outlines the objectives, hypotheses, and includes a literature review focusing on the roles of transferrin, and transferrin-like molecules in acute inflammation. The literature review also focuses on phagocyte-mediated induction, effector mechanisms, and resolution of acute inflammation. The second chapter provides a detailed description of the methodologies used throughout this thesis. In chapter 3 I examined the ability of cleaved transferrin products to serve as a broader marker of the acute inflammatory response in teleost fish. In chapter 4 I investigated the production of cleaved transferrin products during acute inflammation *in vivo*. In chapter 5 I describe my general findings, provide a model of immune transferrin during acute inflammation, provide suggestions for further work that would further characterize this model, and discuss the relevance of my findings to the greater scientific community. I then list all of the references cited at the end of the thesis.

## 1.4 Literature review

### 1.4.1 Transferrins: highly conserved multifunctional glycoproteins

#### 1.4.1.1 Introduction

Transferrins are a highly homologous family of proteins that posses a diverse array of physiological roles throughout evolution. Most transferrins are 70 – 80 kDa bi-lobed proteins that consist of homologous N- and C-lobes. In vertebrates known members of the transferrin family consist of serum transferrin, lactotransferrin, ovotransferrin, melanotransferrin, and the inhibitor of carbon anhydrase (Dunn et al., 2006; Farnaud and Evans, 2003; Lambert et al., 2005; Metz- Boutigue et al., 1984; Wuebbens et al., 1997). However transferrin proteins are not exclusive to vertebrates with transferrin homologues

found in echinoderms, arthropods, and even as distantly as marine algae (Lambert, 2012; Lambert et al., 2005).

Historically, transferrins have been well characterized for their role in iron binding and transport. However transferrins also play a significant role in modulating the immune system. Indeed, there is evidence of the evolution of transferrin as an immunoregulatory molecule, as well as evidence of pathogens attempts to manipulate host transferrin for their own benefit. For example: transferrin contributes to host defence: a) by sequestering iron from invading pathogens (Ellis, 2001; Skaar, 2010), b) as an acute phase protein (Bayne and Gerwick, 2001; Cray et al., 2009; Kovacevic et al., 2015), c) through direct killing of bacterial pathogens (Ibrahim et al., 1998), and d) by activating anti-microbial responses in macrophages *in vitro* (Jurecka et al., 2009; Stafford and Belosevic, 2003; Stafford et al., 2001). Transferrin degradation products have been found in the bronchoalveolar lavage fluid of cystic fibrosis patients infected with *Pseudomonas aeruginosa* (Britigan et al., 1993) and there are several pathogens that produce a transferrin-receptor like protein capable of binding holo-transferrin (Cornelissen, 2003).

Although there are several excellent reviews on the role of transferrin in iron metabolism, see:(Dautry-Varsat et al., 1983; Gkouvatsos et al., 2012; Harding et al., 1983; Johnson and Wessling-Resnick, 2012), the roles of transferrins in immunity have not been as thoroughly discussed. In this section I will highlight the recent advances in our understanding of the immunoregulatory roles of transferrin, with an emphasis on their role in the acute inflammation.

### 1.4.1.2 Transferrin and transferrin receptor structure

Transferrin is a bi-lobed monomeric glycoprotein, of approximately 70 – 80 kDa**.** The human serum transferrin consists of 679 amino acids and two N-liked oligosaccharide chains. The protein folds into two homologous domains coned the N- and C-lobes conntected by a small ~15 residue peptide interdomain bridge, each capable of finding one iron molecule (Figure 1.1A). Both glycosylation sites are located in the C-lobe of the molecule. The protein is composed of several α-helices and β-sheets (Figure 1.1B).

The transferrin receptor is two-subunit transmembrane glycoprotein of approximately 180 kDa. The human transferrin receptor consists of two 760 amino acid polypeptide chains held together through di-sulphide bonds (Figure 1.1C). The homodimer is able to bind two molecules of transferrin molecules and bring them into the cell (Schneider et al., 1984).

### 1.4.1.3 Transferrin in iron storage and transport

Transferrin has been well characterized for its role in vertebrate iron transport (Gkouvatsos et al., 2012). In vertebrates, apo-transferrin is primarily produced by the liver and released into the blood stream (Zakin, 1992). Once in the serum each transferrin molecule will tightly bind two free iron molecules (Aisen P Fau - Leibman et al., 1978). Once bound to iron holo-transferrin will bind to transferrin receptors on the plasma membrane, and is taken into the cell by receptor mediated endocytosis at a rate of approximately 18 000 holo-transferrin molecules per minute per cell, and each cell containing approximately 200 000 transferrin receptors (Dautry-Varsat et al., 1983; Sawyer and Krantz, 1986). During endosome maturation the decrease in pH causes

transferrin to release the bound iron molecules, and transferrin is recycled back into circulation (Dautry-Varsat et al., 1983). An iron that is not used immediately in metabolic processes is then bound to ferritin, an intracellular iron storage protein (Harrison and Arosio, 1996). Despite recycling of transferrin molecules, transferrin has a relatively short life span with humans turning over their entire transferrin pool every 3 hours (Cavill, 2002). Only approximately 0.1% of total iron is bound to transferrin, however transferrin-bound iron it the most accessible and plastic form of iron throughout the body (Wang and Pantopoulos, 2011).

#### 1.4.1.4 Evolution of non-vertebrate transferrins

The transferrin superfamily of proteins encompasses the well-characterized vertebrate transferrins mentioned earlier; however it also includes several other homologues such as: saxiphilin in amphibians (Li and Moczydlowski, 1991), major yolk protein in hemichordates (Brooks and Wessel, 2002), insect transferrin (Yoshiga et al., 1999), and even a tri-lobed transferrin like molecule found in marine algae (Fisher et al., 1997; Fisher et al., 1998). Many of these homologues have developed various physiological roles and tissue specific expression patterns.

*1.4.1.4.1. Marine-algae transferrin like molecules*

Marine algal species of the genus *Dunaliella* are found in hypersalinic environments and produce a 150 kDa tri-lobed transferrin like molecule (Fisher et al., 1997). This protein is localized to the plasma membrane and facilities iron acquisition from the environment. This transferrin-like molecule also exhibits several characteristics similar to vertebrate transferrin, including preferential binding of $Fe^{3+}$ iron species over $Fe^{2+}$, reliance on carbonate/bicarbonate ions, and expressional plasticity where transferrin

is up-regulated in low iron environments and down-regulated in high iron environments (Fisher et al., 1997; Fisher et al., 1998; Paz et al., 2007).

*1.4.1.4.2 Insect transferrin*

Insect transferrin has been described in many species including *Drosophila melanogaster* (Yoshiga et al., 1999), *Aedes* species, (Yoshiga et al., 1997), termites (Thompson et al., 2003), beetles (Kim et al., 2008), honeybees (Kucharski and Maleszka, 2003), and moths (Yun et al., 2009). Similar to vertebrate transferrin insect transferrin is bi-lobed, found in the hemolymph, and also display localized synthesis in the fat body; which is equivalent to the vertebrate liver. However recent studies suggest that in some species the C-lobe has lost the ability to bind iron, but has gained immune functions. Insect transferrin is an acute phase protein, being up-regulated in response to bacterial pathogens (Yoshiga et al., 1999; Yoshiga et al., 1997), and a recombinant moth, *Bombyx mori*, transferrin directly inhibits bacterial growth (Yun et al., 2009). To date, insects are one of the earliest known evolutionary lineages that use transferrin as an immune molecule.

*1.4.1.4.3 Major yolk protein*

In sea urchins, echinoderms, their eggs contain a transferrin homolog called major yolk protein. This protein is produced in the ovaries, testis, coelomic fluid, and intestine of males and females (Brooks and Wessel, 2002; Unuma et al., 2010; Unuma et al., 2003). Major yolk protein has been shown to bind iron, similar to vertebrate transferrin, however the specific binding stoichiometry has not been elucidated as it has in insects and vertebrates (Brooks and Wessel, 2002). In some species of sea urchin, specifically *Paracentrotus lividus* and *Tripneustes gratilia*, the major yolk protein has been modified

to form a distinct homolog known as the toposome (Noll et al., 2007). Similar to major yolk protein the toposome is a major component of sea urchin eggs, however it plays significant roles in calcium binding and cell adhesion (Noll et al., 2007). Although the toposome lacks several conserved residues that are critical in iron binding of other transferrin homologues the toposome has been shown to bind $Fe^{3+}$ suggesting multiple roles for these transferrin homologues in echinoderms (Hayley et al., 2008).

#### 1.4.1.5 Vertebrate transferrins

Transferrin has been well characterized in all vertebrate lineages, including teleost fish, amphibians, reptiles, birds, and mammals. Many of the diverse roles of transferrin were first elucidated in non-mammalian vertebrate species, for example the ability of transferrin cleavage products to induce anti-microbial responses in macrophages was initially discovered in a teleost fish model, *Carassius auratus* (Stafford et al., 2001). Transferrin has also been identified in several other teleost species, including rainbow trout (Murayama et al., 2000), sockeye salmon (Møller, 1970), and medaka (Mikawa et al., 1996) and is likely present in all teleosts.

##### *1.4.1.5.1 Amphibian transferrins*

Transferrin has also been well characterized in several amphibian models, such as the African Clawed Frog, *Xenopus laevis*, and North-American bullfrog, *Rana catesbiana* (Llewellyn et al., 1997; Morabito and Moczydlowski, 1995). It is important to note that recently, a non-iron binding transferrin homolog, saxiphilin, has been identified in *R. catesbiana* but this has yet to be identified in *Xenopus* genomes (Llewellyn et al., 1997). This *Rana* saxiphilin shares a high degree of similarity with *Xenopus* transferrin, with the N- and C-lobes having 50% and 57% similarity (Lambert, 2012; Lambert et al., 2005).

*1.4.1.5.2 Ovotransferrin*

Reptiles and birds both posses a serum transferrin homolog known as ovotransferrin. This molecule was first characterized in egg whites demonstrating both iron-transport characteristics and as an inhibitor of microbial growth, similar to insect transferrins (Schade and Caroline, 1944). Ovotransferrin is also found in the serum, being the primary iron transport molecule in the blood (Giansanti et al., 2012). Furthermore it has been shown that ovotransferrin can participate in direct killing of the bacteria *Staphlococcus aureus* (Ibrahim et al., 1998; Ibrahim et al., 2000; Xie et al., 2002b). However the specific mechanism of bacterial killing by Ovotransferrin is unknown.

*1.4.1.5.3 Lactoferrin and the inhibitor of carbonic anhydrase*

In mammals neither lactoferrin nor the inhibitor of carbonic anhydrase play a major role in iron homeostasis, with lactoferrin knockout mice showing no variation in iron homeostasis, intestinal development, or fertility (Ward et al., 2003). The inhibitor of carbonic anhydrase was originally found in pig serum where it has completely lost its ability to bind iron; instead its only characterized role is as an inhibitor of carbonic anhydrase (Roush and Fierke, 1992; Wuebbens et al., 1997). In primates the inhibitor of carbonic anhydrase gene contains premature stop codons meaning the inhibitor of carbonic anhydrase is likely a pseudogene (Schaeffer et al., 1987).

*1.4.1.5.4 Melanotransferrin*

Melanotransferrin is commonly associated with human melanoma tumours, however is also expressed in normal tissues including the salivary glands, sweat glands, liver, and intestine. Only the N-lobe of melanotransferrin is capable of binding iron

(Baker et al., 2002) and similar to lactoferrin does not play a critical role in iron homeostasis with melanotransferrin knockout mice showing no phenotypic change in iron transport (Dunn et al., 2006; Sekyere et al., 2006).

#### 1.4.1.6 Immune roles of transferrin

##### *1.4.1.6.1 Transferrin is an acute phase protein*

The acute phase response (APR) is a prominent physiological response of a host to injury, trauma, or infection. This orchestrated systemic response involves physiological changes in several host organ systems (Baumann and Gauldie, 1994; Bayne and Gerwick, 2001; Gruys et al., 2005). Specifically the acute phase response involves changes in the hepatic, musculo-skeletal, central nervous, and immune systems. Common symptoms of the acute phase response include: fever, pain, leucocytosis, and increased synthesis of proteins classified as acute phase proteins. Although there are several acute phase proteins, with variation between species, transferrin is a protein commonly associated with change during infection (Feelders et al., 1998). These changes are dependent on species with transferrin commonly being associated as a negative acute phase protein (Cray et al., 2009), however has shown to be a positive acute phase protein in other animals such as chickens (Bayne and Gerwick, 2001; Xie et al., 2002a) and trout In response to pathogens transferrin gene expression is increased both locally by macrophages in several evolutionary lineages (Djeha and Brock, 1992; Djeha et al., 1995; Stafford and Belosevic, 2003; Stafford et al., 2001; Yoshiga et al., 1999; Yoshiga et al., 1997), and in some vertebrates, globally by liver hepatocytes (Bayne and Gerwick, 2001; Cray et al., 2009; Kovacevic et al., 2015; Xie et al., 2002a; Xie et al., 2002b; Xie et al., 2002c).

*1.4.1.6.2 Transferrin sequesters iron from invading pathogens*

The battle between pathogens and their hosts has received significant attention in the literature due to several strategies hosts have adapted to restrict iron from invading pathogens, and conversely, pathogen strategies to maximize iron availability (Doherty, 2007; Schaible and Kaufmann, 2004; Skaar, 2010). Iron is initially inaccessible to infiltrating pathogens as the majority of iron in healthy individuals is intracellular, and the minute amount of extracellular iron is tightly bound to transferrin or lactoferrin (Bullen et al., 1999; Kehl-Fie and Skaar, 2010). In a further attempt to sequester iron macrophages increase transferrin expression and decrease transferrin receptor expression in response to inflammatory stimuli. Current evidence suggests this strategy prevents iron acquisition by intracellular pathogens, and allows iron to be tightly bound to transferrin rather than weakly bound to intracellular ferritin (Byrd and Horwitz, 1989; Hamilton et al., 1984; Ponka and Lok, 1999).

*1.4.1.6.3 Transferrin is involved in leucocyte differentiation and maturation*

It has been well established that proliferating cells have a critical requirement for iron, and thereby, transferrin (Le and Richardson, 2002; Rossi and Zetter, 1992; Vostrejs et al., 1988). If iron is restricted cell growth stalls, specifically preventing cells progressing from the $G_1$ to M phase of cell division (Laskey et al., 1988). However it has been shown that transferrin molecules also play a significant role in leucocyte differentiation and maturation, in addition to satisfying these leucocytes iron requirement. Recently it has been shown that transferrin is required for T-cell differentiation events in the thymus, prior to TCR-mediated selection events (Macedo et al., 2004; Macedo and

Sousa, 2008). It has also been reported that transferrin contributes to granulocyte end-stage maturation events (Evans et al., 1989; Evans et al., 1986).

*1.4.1.6.4 Transferrin can induce pro-inflammatory responses*

Transferrin molecules have been shown to act directly on cells by enhancing anti-microbial responses. Transferrin can increase chemokine expression in human epithelial cells, and increase iNOS expression in smooth muscle cells (Takenaka et al., 1995; Takenaka et al., 2000; Tang et al., 2002). Transferrin has been shown to be a component of platelet secretions that enhance phagocytosis (Sakamoto et al., 1997). Recently it has been shown that endogenously cleaved transferrin fragments can induce anti-microbial responses in goldfish and murine macrophages *in vitro* (Haddad and Belosevic, 2009; Jurecka et al., 2009; Stafford and Belosevic, 2003; Stafford et al., 2001; Stafford et al., 2004). The ability of these cleavage products is highly conserved with both bovine and teleost cleavage products having the capacity to enhance the anti-microbial responses to several groups of pathogens in both teleost and murine macrophage systems (Haddad and Belosevic, 2009).

*1.4.1.6.5 Cleaved transferrin products in the induction of pro-inflammatory responses*

Recently it has been shown that cleaved transferrin has a potent ability to induce anti-microbial responses in macrophages (Stafford and Belosevic, 2003; Stafford et al., 2001). This phenomenon was initially identified during *in vitro* culture of mitogen-activated mixed leucocyte cultures, and the presence of fragmented bovine and teleost transferrin in cell culture supernatants. These transferrin fragments were then purified and applied to *in vitro* derived kidney macrophages were shown to directly induce the production of nitric oxide (Stafford et al., 2001). Furthermore it has been shown that

addition of transferrin cleavage products will enhance the NO response of macrophages in response to stimulation with pathogens alone (Stafford et al., 2003).

The generation of the immunostimulatory transferrin molecules is shown to arise from endogenous enzymes produced by leucocytes during *in vitro* cell culture (Stafford and Belosevic, 2003; Stafford et al., 2001). Specifically, macrophages have been shown to produce transferrin cleavage products *in vitro* (Jurecka et al., 2009). The cleavage site was initially identified to be within the interdomain bridge (Stafford and Belosevic, 2003), however this likely represents an initial cleavage event during a cascade of several cleavage subsequent events given the plethora of transferrin breakdown products identified and the ability of several different enzymes being able to facilitate transferrin cleavage (Haddad and Belosevic, 2009) (Table 1.1). Interestingly, several transferrin variants are expressed that have been shown to have differential capacity to induce NO production in macrophages (Jurecka et al., 2009). However, despite the difference between transferrin isoforms a specific 31 residue transferrin-derived peptide, present in all isoforms, induces NO responses in murine and teleost macrophages (Haddad and Belosevic, 2009). This peptide was shown to be derived from the N-lobe, contained six cysteine residues and resembles a defensin molecule, however was not able to act directly against either gram negative or positive bacteria suggesting the primary role of this peptide is to induce leucocyte-mediated anti-microbial responses rather than act directly against pathogens (Haddad and Belosevic, 2009). Currently the specific receptor that facilitates the induction of anti-microbial responses upon binding of cleaved transferrin products is unknown. Other endogenous molecules capable to stimulating immune responses have been suggested to stimulate through the NF-κB/TLR receptor system (Li

et al., 2001), making an unidentified TLR an excellent candidate for exerting the anti-microbial activity of cleaved transferrin products.

#### 1.4.1.7 Immune roles of transferrin family members

##### *1.4.1.7.1 Lactoferrin in response to infection*

Lactoferrin is found in several secretory fluids including: milk, saliva, tears, bile, vaginal secretions, and seminal fluid, among others (García-Montoya et al., 2012). Interestingly, lactoferrin is also produced and stored in granulocytes (Breton-Gorius et al., 1980; Faurschou and Borregaard, 2003). Apoptotic cells, but not necrotic cells, release lactoferrin inhibiting granulocyte migration and simulating monocyte migration to enhance resolution mechanisms during acute inflammation (Bournazou et al., 2009; Poon et al., 2014). Although lactoferrin shares many similar iron-binding properties with transferrin, it is not a critical component of iron transport and metabolism (Ward et al., 2003). Instead lactoferrin is believed to enhance effector mechanisms of the innate immune system. Lactoferrin is able to bind iron more tightly, and over a broader range of pH, than transferrin helping it sequester iron from invading pathogens (Choe et al., 2003; Ward and Conneely, 2004). Lactoferrin is also able to act directly against microorganisms by containing anti-microbial peptides, released during protein digestion (van der Kraan et al., 2004).

##### *1.4.1.6.2 Ovotransferrin in immunity*

Ovotransferrin is the mammalian serum transferrin equivalent in avian and reptilian species, and is also found prominently in egg whites (Giansanti et al., 2012; Schade and Caroline, 1944). Ovotransferrin shares similar immunological properties as both transferrin and lactoferrin. Ovotransferrin is a positive acute phase protein in

chickens, being up-regulated in response to infection with viruses, bacteria, and other inflammatory agents. Ovotransferrin has been shown to modulate reactive oxygen intermediates (ROI) and reactive nitrogen intermediates (RNI) species in avian macrophages and heterophils (Xie et al., 2002b; Xie et al., 2001). Similar to lactoferrin, ovotransferrin has anti-bacterial peptide sequences that inhibit bacterial growth when the native protein is degraded (Ibrahim et al., 1998; Ibrahim et al., 2000).

*1.4.1.6.3 Other transferrin molecules*

The immunological roles of transferrin homologues in other species have not been as well investigated. In several insect species it has been shown that transferrin expression increases in response to infection, acting as a positive acute phase protein (Yoshiga et al., 1999; Yoshiga et al., 1997). A recombinant moth, *B. mori*, transferrin has been shown to directly inhibit bacterial growth, however the specific mechanism of bacterial inhibition remains unclear (Yun et al., 2009). Insects have a ferritin homolog which is secreted (Dunkov and Georgieva, 2006; Geiser et al., 2006). Current evidence suggests this secreted iron storage protein plays the primary role of iron transport, storage, and sequestration in insects rather than the insect transferrin, making the primary role of insect transferrin as an immune molecule.

**1.4.1.8 Pathogen manipulation of transferrin molecules**

Iron is an essential component of pathogen growth and proliferation within a host. I have outlined strategies hosts use to sequester iron from invading pathogens however pathogens have developed several effective methods to circumvent the issue of a limited iron supply within a host. Although there are several strategies used by pathogens to

acquire iron, and many pathogens employ more than one of the discussed strategies, I will focus only on those that have a direct relationship to transferrin.

*1.4.1.8.1 Sidrophores*

The production of pathogen-derived siderophores is a common iron acquisition strategy used by bacteria, protozoans, and several fungi. Sidreophores are small (<1 kDa), secreted, iron scavenging proteins that will tightly bind any free iron, and can also scavenge iron directly from transferrin, making the iron available to pathogen metabolism (Saha et al., 2013; Weinberg, 2009). There are several different siderophores produced, possessing several diverse mechanisms for acquiring transferrin-bound iron. One strategy employed by *Pseudomonas aeruginosa* is that the bacteria will cause a decrease in pH of the microenvironment, causing the iron-transferrin affinity to decrease while the siderophore-iron binding affinity remains high (Sriyosachati and Cox, 1986). An alternate siderophore-mediated strategy, employed by the fungus *Aspergillus fumigatus*, is the production of siderophores with a higher iron-binding affinity than host transferrin (Hissen et al., 2004).

*1.4.1.8.2 Internalization of transferrin*

One such strategy employed by diverse pathogen groups is the direct uptake of holo-transferrin. Gram-negative bacteria of the genera *Neisseria* (Mickelsen and Sparling, 1981), *Moraxella* (Campagnari et al., 1994), *Actinobacillus* (Gerlach et al., 1992), and *Haemphilus* (Herrington and Sparling, 1985) express a receptor with the specificity to directly bind and internalize holo-transferrin, but not apo-transferrin. The protozoan pathogen *Trypanoplasma boreii*, a common blood parsite of carp, is able to actively take up host proteins in the flagellar pocket and digest them in a lysosomal compartment

(Ruszczyk et al., 2008). Other members of the Trypanosomatida, which include *Trypanosoma* and *Leishmania* species, manipulate transferrin in a similar manner (Lima and Villalta, 1990; Schell et al., 1991; Steverding, 2000; Voyiatzaki and Soteriadou, 1992).

*1.4.1.8.3 Degradation of transferrin*

An interesting strategy used by several pathogens is degrading full-length transferrin, making in unable to bind iron. Pathogens can either secrete enzymes that directly cleave transferrin, such as the case with *Pseudomonas aeruginosa*, which secrets elastase that degrades transferrin (Britigan et al., 1993; Britigan et al., 1992), or some pathogens will degrade transferrin in a lysosome and then release these fragments back into the host, as is the case with *Trypanoplasma boreii* (Ruszczyk et al., 2008). This strategy is intriguing due to the ability of cleaved transferrin products to induce pro-inflammatory responses in macrophages (Haddad and Belosevic, 2009; Jurecka et al., 2009; Stafford and Belosevic, 2003; Stafford et al., 2001). Indeed, as mentioned earlier, endogenous enzymes can produce transferrin cleavage products, as well as the contents from lysed, but not apoptotic cells. This poses an interesting paradigm for the evolution of acute inflammation, where a pathogen attempt to manipulate its host either by degrading transferrin, or by inducing leucocyte necrosis, these events can then induce acute inflammation.

### 1.4.2 Effector mechanisms and mediators of acute inflammation in teleost fish

#### 1.4.2.1 Introduction

Inflammation is a tightly regulated and intricate process that is crucial for effective mitigation of pathogen invasion and tissue damage. Effective control of

inflammation is crucial to restore and maintain homeostasis following tissue injury or pathogen infiltration. Inefficient induction mechanisms can often lead to hosts being overrun by infectious agents or developing sepsis (Wong and Billiar, 1994). Poor regulation of resolution mechanisms can often lead to chronic inflammatory conditions such as: systemic lupus erythematous, rheumatoid arthritis, and Crohn's disease, among others (Buckley et al., 2001).

Acute inflammation is characterized by the rapid induction of inflammatory cytokines, subsequent recruitment of phagocytic leucocytes, and induction of anti-microbial responses – localized to the inflammatory site (Serhan and Savill, 2005). These characteristics of acute inflammation are highly conserved, critical components, of the innate immune system. Many of the hallmark characteristics have been characterized in mammals, and non-mammalian vertebrates and non-vertebrates share a high degree of homology to the mammalian acute inflammatory response. Importantly, many non-vertebrates, display a much greater reliance on innate mechanisms in the absence highly specialized classical acquired immune mechanisms (Medzhitov, 2001). Mammals have a much greater reliance on a highly specialized acquired immune response, not seen in non-vertebrate lineages (Yang et al., 2002). Teleost (bony) fish are the earliest evolutionary lineage to display classical acquired immunity (Uribe et al., 2011), making them an excellent model to study the initial mechanisms of acute inflammation and examine their potential role in tailoring adaptive responses, characteristic of the acquired immune system.

### 1.4.2.2 Leucocytes involved in acute inflammation

Leucocytes are the primary cells responsible for initiating, coordinating, and resolving an effective immune response to damaged tissue or pathogens. Leucocyte responses during acute inflammation have been well characterized in mammalian systems, and will be highlighted during this section. However I will integrate recent complimentary literatures advances in our understanding of teleost leucocyte roles in acute inflammation when information is available. Many of the roles of teleost leucocytes will also be discussed during the discussion of results in later chapters, where I will also highlight the divergent roles of teleost and mammalian leucocytes in effective inflammatory-control.

#### *1.4.2.2.1 Mononuclear cells*

Monocytes and macrophages are critical for effective induction, effector mechanisms, and resolution of acute inflammation. The initial induction of an inflammatory response is triggered by resident macrophages recognizing damaged tissues, or pathogens and/or pathogen products. These inflammatory triggers are also known as damage-associated molecular patters (DAMPs) or pathogen-associated molecular patterns (PAMPs) (Kawai and Akira, 2010). Binding of DAMPs or PAMPs to pattern recognition receptors (PRRs) on resident macrophages results in the rapid production or pro-inflammatory cytokines that result in leucocyte recruitment and further activation events. In teleost fish these events include production of the neutrophil recruiting chemokine CXCL-8, production of pro-inflammatory cytokines TNF-α, IL-1β and IFN-γ (Havixbeck et al., 2016; Rieger et al., 2015). The initial infiltrating leucocyte population is neutrophils, which will be detailed in the next section of this review.

During the primary effector phase of acute inflammation there are a plethora of macrophages and monocytes that participate in anti-microbial response to combat pathogens. Macrophages and monocytes have been well characterized for their anti-microbial activity, employing several strategies such as: phagocytosis, respiratory burst (ROS), nitric oxide production (NO), and phagolysosome fusion (Neumann et al., 2000). These mononuclear cells displays marked differences in their capacity for each of the listed anti-microbial responses. Monocytes display reduced ability to generate ROS and NO species, however they have a greater capacity for phagolysosome fusion (Neumann et al., 2000; Rieger et al., 2012).

After the removal of the pathogenic stimuli macrophages and monocytes facilitate the resolution phase by removing any damaged tissues, and apoptotic cells. Although recently it has been shown that mammalian neutrophils have the capacity to internalize apoptotic cells (Esmann et al., 2010), and teleost neutrophils may play a regulatory role during resolution (Havixbeck et al., 2016), mononuclear cells still play a prominent role in the orchestration of effective resolution mechanisms. The resolution phase is typically characterized by high levels of TGF-β and IL-10, combined with a decrease in pro-inflammatory cytokines TNF-α, IL-1β, IFN-γ, and intracellular killing mechanisms ROS and NO (Rieger et al., 2015; Rieger et al., 2012). It is also characterized by increased uptake of apoptotic cells by mononuclear cells (Poon et al., 2014).

*1.4.2.2.2 Neutrophils*

Neutrophils are one of the primary cells types affiliated with pathogen killing. Neutrophils posses a robust array of intra- and extracellular anti-microbial killing mechanisms employed during acute inflammation. Similar to macrophages neutrophils

are capable of employing phagocytosis and then producing intracellular ROS and NO. Interestingly, neutrophils also posses a marked capacity to produce extracellular ROS and NO to combat extracellular pathogens not seen in macrophages (Forlenza et al., 2008; Katzenback and Belosevic, 2009).

A hallmark strategy of neutrophil anti-microbial responses is the release of intracellular storage granules, degranulation. These storage granules contain a plethora of cytotoxic products, that include: elastase, defensins, lysozyme, myeloperoxidase, hydrolases, and many other anti-microbial substances. These granules can be released directly into intracellular lysosomes, or extracellularly (Farnaud and Evans, 2003). The release of these intracellular storage granules also facilities the formation of neutrophil extracellular traps (NETs). NETs are composed of these granule proteins, complexed with DNA fibres that aid in the capture and directly killing of bacteria (Yipp et al., 2012).

This unique ability of neutrophils to utilize these extracellular killing mechanisms also can have a deleterious effect on host tissues. Although these non-specific extracellular responses are extremely effective at pathogen killing, they also damage host tissue. Indeed, neutrophils have been well characterized for the gastrointestinal damage associated with non-steroidal anti-inflammatory medications (NSAIDs), such as ibuprofen (Wallace et al., 1990; Yoshikawa and Naito, 2000). These anti-inflammatory drugs inhibit COX-2, which is primarily responsible for producing the lipid mediator $PGE_2$, and coordinating down-stream effector pathways to reduce pro-inflammatory neutrophil mediated mechanisms (Harris et al., 2002; Ricciotti and FitzGerald, 2011).

#### 1.4.2.3 Roles of non-immune tissues during acute inflammation

Although leucocytes are the primary cells associated with facilitating immune responses there are several non-immune cells and tissues that also participate in host defence. In particular the adherent epithelial cells can are able to induce immune responses, and also participate in during immune effector mechanism (Gewirtz et al., 2001; Poon et al., 2014). One of the hallmark events of inflammation, mediated by a typically non-immune tissue, is the increase in vascular permeability by endothelial cells to allow the influx of leucocytes and serum proteins into the inflammatory site (Pober and Cotran, 1990). In addition to the vasodilation the endothelial cells are also able to secret a variety of prostaglandins, chemokines, cytokines, and proteases into the inflammatory site (Pober and Cotran, 1990; Gewitz et al., 2001). Furthermore endothelial cells are also able to participate in immune effector mechanisms, being able to phagocytose both pathogenic and homeostatic particles (Hamill et al., 1986; Poon et al., 2014).

#### 1.4.2.4 Anti-microbial leucocyte effector mechanisms

##### *1.4.2.4.1 Respiratory burst*

Respiratory burst is a well-characterized phagocyte mediated-response to pathogens or stimulation with pro-inflammatory cytokines. The process of ROS production during respiratory burst is primarily mediated by NADPH-oxidase. NADPH-oxidase is an enzyme complex that is localized to the inner surface of the plasma membrane (Boltana et al., 2009; Mayumi et al., 2008). Production of ROS is essential for intracellular killing within phagolysosomes (Newman, 1999). Indeed, a genetic mutation in any of the NADPH-oxidase subunits can result in increased susceptibility to bacterial and fungal infections (Heyworth et al., 2003).

As mentioned earlier neutrophils also have the capacity to produce extracellular ROS. In addition to the role of intracellular ROS in killing of internalized microorganisms, extracellular ROS contributes to the killing of extracellular pathogens (Broeg and Steinhagen, 2011; Forlenza et al., 2008; Leiro et al., 2001)

*1.4.2.4.2 Nitric oxide*

Nitric oxide is a highly conserved molecule with many diverse physiological functions. Nitric oxide acts as a signalling molecule or a cytotoxic chemical, is generated by several cell types (Lincoln et al., 1997). Synthesis of nitric oxide is mediated by the enzymes nitric oxide synthase (NOS), which convert L-arginine to NO and L-citrulline, utilizing electrons donated by NADPH oxidase (Nathan and Xie, 1994). NOS encompass three isotypes: the constitutively expressed neuronal NOS (nNOS), and endothelial NOS (eNOS), and the immunology relevant isoform: inducible NOS (iNOS). The two constitutive isoforms are able to rapidly produce small amounts of nitric oxide for a short period of time. Whereas iNOS is able to produce larger quantities of NO for a longer period of time. Production of these large amounts of NO requires *de novo* iNOS synthesis by leucocytes, delaying the NO response compared to respiratory burst. Indeed, as mentioned earlier NO synthesis is linked to the electron donating activity of NADPH oxidase. This indicates the respiratory burst and NO responses are linked with NO responses, typically increasing as respiratory burst responses decrease (Neumann, 1999).

Similar to the respiratory burst response, NO production is induced by stimulation with PAMPs or pro-inflammatory cytokines. However, in addition to the pro-inflammatory stimuli, transferrin cleavage products have also been shown to induce high-levels of NO production, and enhance the NO response of macrophages to various

pathogens *in vitro* (Jurecka et al., 2009; Stafford and Belosevic, 2003; Stafford et al., 2001). Macrophages will synthesize NO that will localize to the phagolysosome to aid in pathogen killing (Bogdan, 2001). Also similar to respiratory burst neutrophils are able to produce extracellular NO (Forlenza et al., 2008).

#### 1.4.2.5 Soluble markers of acute inflammation

##### *1.4.2.5.1 TNF-α*

Tumour necrosis factor (TNF)-α is a pleiotropic cytokine that has major roles in the induction and propagation of acute inflammation. TNF-α is one of the earliest markers of the induction phase of inflammation, being produced by resident macrophages in response to pathogens and pathogen products. Indeed, production of TNF-α by murine macrophages increases significantly as early as 1 hour post-stimulation and decreases quickly, back to homeostatic levels by 6 hours (Cui et al., 2000). Other pro-inflammatory cytokines such as IL-1 and IL-6 also induce expression of TNF-α by macrophages. Due to its diverse array of physiology effects TNF-α has, historically, been a widely used marker of acute inflammation. A few of the immunological effects of TNF-α include promotion of phagocyte recruitment (Ming et al., 1987), phagocytosis enhancement (Lohmann-Matthes et al., 1991), and facilitating increased respiratory burst and NO responses (Ding et al., 1988).

##### *1.4.2.5.2 IL-1β*

Interleukin (IL)-1β is another prototypical pro-inflammatory cytokine produced during acute inflammation. The primary function associated with IL-1β is the induction of fever, thus IL-1β is also commonly referred to as an endogenous pyrogen (Harden et

al., 2008). IL-1β is usually produced by mononuclear cells as a pro-peptide and undergoes caspase cleavage into its active form by PRR-mediated signals (Underhill and Goodridge, 2012). The pro-IL-1β is expressed in response to pathogens, and pathogen-associated products, showing similar down-stream effects to TLR-signalling (Janssens and Beyaert, 2002). Although IL-1β has several diverse physiological effects, during acute inflammation IL-1β induces synthesis of lipid mediators such as $PGE_2$, promotes leucocyte chemotaxis, and increases endothelial receptors that mediate diapadesis (Dinarello, 2011)

*1.4.2.5.3 IFN-γ*

Interferon (IFN)-γ is a member of the type II interferon class of cytokines and has several immunological roles in acute inflammation, as well as in the adaptive immune response. Initially, IFN-γ was characterized as a lymphocyte (CD4+, CD8+, and NK-cells) produced cytokine, however recently it has been shown that mononuclear cells also produce significant amounts of IFN-γ. Production of IFN-γ is important for the initial autocrine and paracrine macrophage activation events during the induction of acute inflammation (Frucht et al., 2001; Young, 1996). IFN-γ has a multitude of effects on mononuclear cells such as increased antigen presentation, increased lysosome activity, and increases in respiratory burst and NO production (Decker et al., 2002). In addition to effects on macrophages IFN-γ also facilitates lymphocyte recruitment and activation, facilitating the development of an acquired memory response to pathogens (Martín-Fontecha et al., 2004; Oppenheimer-Marks and Ziff, 1988).

*1.4.2.5.4 TGF-β*

Transforming growth factor (TGF)-β is an interesting cytokine that has historically been used as an anti-inflammatory marker. However TGF-β has also been shown to exert several pro-inflammatory effects. In mammals TGF-β is released rapidly by platelets following an immune challenge acting as a leucocyte chemoattractant (Adams et al., 1991; Reibman et al., 1991; Wahl et al., 1987). At low concentrations TGF-β also increases expression of pro-inflammatory cytokines TNF-α and IL-1 (Allen et al., 1990; Wiseman et al., 1988).

In addition to its pro-inflammatory characteristics TGF-β also exerts several effects that promote resolution of acute inflammation. At higher concentrations TGF-β reduces TNF-α and IL-1β expression by monocytes, decrease's antigen presentation, reduces the anti-microbial respiratory burst, and NO responses (Martinez et al., 2007; Wahl et al., 1988). TGF-β also has immunosuppressive effects on the lymphocyte lineage, inhibiting activation and proliferation. TGF-β directly inhibits mitosis of proliferating lymphocytes, preventing them from progressing from $G_1$ of the cell cycle (Ruegemer et al., 1990; Siepl et al., 1988).

*1.4.2.5.5 IL-10*

Interleukin (IL)-10 exerts a plethora of anti-inflammatory effects on myeloid cells, neutrophils, and lymphocytes. The multitude of anti-inflammatory effects has made it a classical marker of the resolution phase during acute inflammation. IL-10 reduces the macrophage expression effects of pro-inflammatory cytokines TNF-α and IL-1β (de Waal Malefyt et al., 1991; Fiorentino et al., 1991). IL-10 also inhibits the migration of leucocytes by reducing expression of neutrophil chemokines, and increasing macrophage

expression of pro-inflammatory cytokines agonists (Berkman et al., 1995; Kopydlowski et al., 1999; Marfaing-Koka et al., 1996). Interestingly, IL-10 enhanced Fcγ-Receptor-mediated phagocytosis of opsonized ligands by macrophages while down-regulating respiratory burst and NO responses (Capsoni et al., 1995; Spittler et al., 1995).

The effects of IL-10 on neutrophils are not as well characterized as the myeloid lineage, with conflicting reports on whether or not neutrophils actively produce IL-10, respond directly to IL-10 stimulation, or if anti-inflammatory activity of IL-10 on neutrophils is an indirect mechanisms through modulation of macrophages (Cassatella et al., 1997; Kasama et al., 1994; Siewe et al., 2006). However treatment of neutrophils with IL-10 inhibits their ability to phagocytose and induce respiratory burst responses in response to bacteria *in vitro* (Laichalk et al., 1996).

### 1.4.3 Summary

Transferrin is a critical component of iron metabolism, however transferrin also has many critical functions in host defence. Many of these functions are conserved throughout evolution illustrating the importance of transferrin, and transferrin homologues, to host defence. Indeed there are constantly evolving host-pathogen interactions in an attempt to manipulate transferrin for their own benefit. However there are still several questions as to the conservation of transferrin functions, particularly with regards to transferrin homologues in non-mammalian vertebrates, and non-vertebrates lineages.

Acute inflammation is a critical component of host defence, especially in non-mammalian vertebrate and more primitive evolutionary lineages. This process is highly regulated to ensure efficient pathogen removal while maintaining host homeostasis. There

is a vast milieu of regulators for the induction, effector, and resolution phases of acute inflammation including phagocytes, and phagocyte-produced soluble mediators. Many of these processes are well understood in mammalian systems, however the ability of cleaved transferrin products to regulate inflammatory processes is a recent discovery that has not been as well investigated. Given the presence of transferrin- or transferrin like molecules, and macrophages- or macrophage-like cells present in almost all organisms cleaved transferrin products may be a highly conserved, primitive mechanism capable of regulating acute inflammation.

**Table 1.1 – Predicted cut sites of human transferrin by selected proteases (from ExPASy peptide cutter)**

| Enzyme | Number of cut sites |
|---|---|
| Chymotrypsin (high specificity) | 61 |
| Chymotrypsin (low specificity) | 151 |
| CNBr | 10 |
| Neutrophil elastase | 109 |
| Pepsin | 189 |
| Protinease K | 323 |
| Trypsin | 79 |

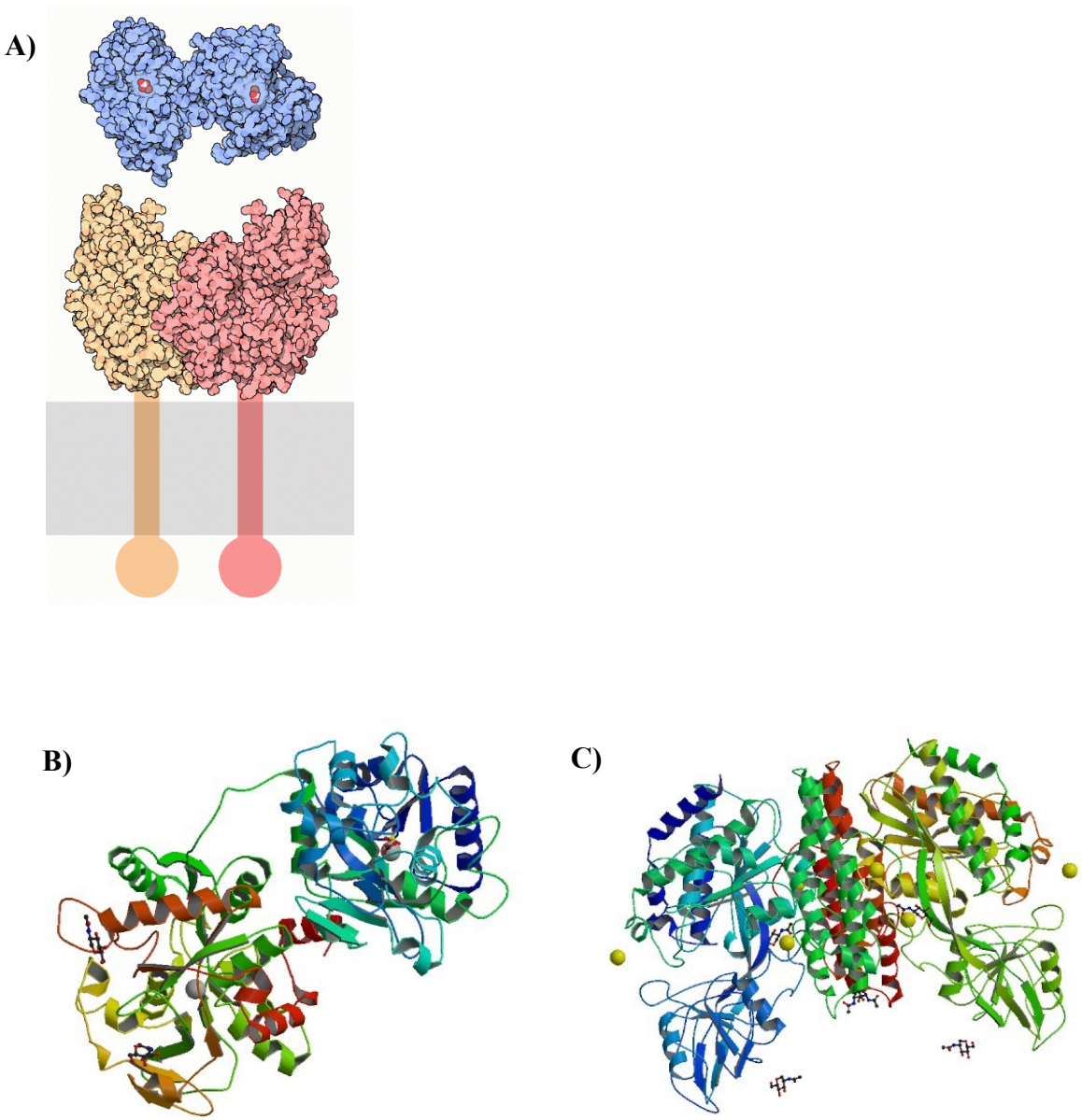



**Figure 1.1 – Schematic diagrams of transferrin and transferrin receptor**. A) Cartoon representation of transferrin and transferrin receptor (from New York Times). B) Ribbon model of transferrin (from ExPASy protein structure predictor (Hass et al., 2013)). C) Ribbon model of transferrin receptor (from ExPASy protein structure predictor (Hass et al., 2013)).

# Chapter 2: Materials and Methods

## 2.1 Animals

Goldfish (*Carrasius auratus* L.) 10 – 15 cm in length were purchased from Mount Parnell (Mercersburg, PA) and maintained in the Aquatic Facility of the Department of Biological Sciences, Edmonton, University of Alberta. The fish were held at 20 °C in a flow-through water system on a simulated local natural photoperiod. Fish were fed to satiation daily with trout pellets and acclimated to this environment for at least 2 weeks prior to use in experiments. Prior to handling, fish were sedated using tricane methane sulfonate (TMS) solution of 40 – 50 $mg \bullet mL^{-1}$. Goldfish were sacrificed via cervical dislocation using approved procedures. Animals were maintained according to the guidelines of the Canadian Council on Animal Care. Protocols were approved by the University of Alberta Animal Care and Use Committee (ACUC-Biosciences: Protocol Number AUP706).

## 2.2 Pathogens and Pathogen Mimics

### 2.2.1 Intraperitoneal injection

Goldfish were anesthetized with TMS. Goldfish were then removed from water and placed on bench-coat. Intraperitoneal injections were done in the soft area under the left pectoral fin. Goldfish were then returned to water with oxygenation, allowed to recover, and monitored for 10 minutes. All injections were performed using a 25-gauge 1 mL needle and all bubbles in the injection solution were removed.

### 2.2.2 Zymosan-induced peritonitis

Zymosan was used to induce acute, self-resolving, inflammation. Injectable zymosan (not particulate) was purchased from Alfa Aesar and resuspended at a concentration of 50 mg•mL$^{-1}$ in 1X PBS$^{-/-}$ (no calcium/no magnesium; see table 1 for composition of 10X solution). Resuspended zymosan was stored at 4 °C until use in experiments. For use in peritoneal injections 2.5 mg of zymosan in 100 µL of 1X PBS$^{-/-}$ was injected intraperitoneally.

### 2.2.3 *Aeromonas veronii*

*Aeromonas veronii* was isolated from a naturally infected goldfish in the Department of Biological Sciences Aquatic Facility by swabbing a body furuncle with a cotton swab and inoculating a trypic soy agar (TSA) plate. Single colonies were grown and confirmed to be *A. veronii* based on sequence analysis. A single colony was used to inoculate trypic soy broth to create a clonal glycerol stock. The growth curve for *A. veronii* can be found in Figure 2.1. Heat-killed *A. veronii* was generated by incubating at 80 °C for 60 minutes with gentle agitation every 15 minutes. Heat-inactivated cultures were centrifuged at 2640 xg for 10 minutes at 4 °C. Ten mL of 1X PBS$^{-/-}$ was then added to wash, and heat-killed cultures were centrifuged again at 2640 xg for 10 minutes at 4 °C. Following heat inactivation, an aliquot of bacteria was plated on TSA plates to confirm that cultures had been killed and no cultures developed. Heat-killed bacteria was stored at 4 °C until further use. For use in peritoneal injection 5.0 x $10^6$ C.F.U. in 100 µL of 1X PBS$^{-/-}$ was injected intraperitoneally.

### 2.2.4 Poly(I:C)-induced inflammation

Poly(I:C) is a double stranded RNA, that acts as a viral mimic. Poly(I:C) (Sigma-Aldrich) was purchased and resuspended in 1X PBS $^{-/-}$ at a concentration of 20 $mg \bullet mL^{-1}$ at stored at -20 °C until further use. To determine an appropriate concentration of poly(I:C) to use for intraperitoneal injection in time course experiments, poly(I:C) was injected at 100, 200, and 400 μg in 100 μL 1X PBS$^{-/-}$ and goldfish were analysed 24 hours post injection. By analysing leucocyte infiltration, respiratory burst, NO production, and the IFN-inducible Mx gene expression I determined 200 μg was appropriate to use in further experiments.

## 2.3 Isolation of goldfish serum

Blood samples were collected from the tail vein of individual goldfish using 23-gauge needle attached to a 1 mL syringe. Blood samples were then immediately placed in BD Vacutainer tubes with clot activator gel for serum separation (BD Biosciences, yellow cap tube) Following collection blood was allowed to clot at room temperature for 10 minutes. Blood collection tubes were then centrifuged at 485 xg for 20 minutes at 4 °C. Serum samples were then collected and stored with protease inhibitor cocktail 8340 (Sigma-Aldrich) at -20 °C until use in further experiments.

## 2.4 Isolation of goldfish leucocytes and supernatants

### 2.4.1 Hema3 staining

Cells were fixed by incubation in Solution I for 1 minute, using the HEMA3 manual staining system (Fisher Scientific), stained in Solution II for 1 minute and counter-stained in solution III for 20 seconds. Excess stain was removed by rinsing with

distilled water, and stains were air-dried prior to observation using bright field microscopy. Photomicrographs were generated using a DM1000 microscope (Leica) using a bright field 100x objective (1000x magnification). Images were acquired using QCapture software.

### 2.4.2 Sudan Black staining

One hundred thousand cells were spun on glass slides at 55 xg for 6 minutes at room temperature using a cytocentrifuge (Shandon Instruments). For Sudan black staining (Sigma), cells were fixed with a 75% gluteraldehyde: 25% acetone fixative solution for 1 minute at 4 °C. Slides were then rinsed well with distilled water and stained with Sudan Black for 5 minutes with continuous, gentle agitation. Slides were thoroughly rinsed with 70% ethanol to remove excess Sudan Black staining and further rinsed in distilled water. Cells were counterstained with Gill's 3 solution (Sigma) for 5 minutes and rinsed with tap water for 2 minutes. Slides were air dried prior to observation using bright-field microscopy as described above.

### 2.4.3 Isolation of kidney leucocytes

Goldfish were anesthetized with TMS and sacrificed by cervical dislocation. The kidney was removed and placed into a petri dish containing ice-cold incomplete medium (MGFL-15) (Tables 2.2, 2.3, 2.4). The kidney was gently homogenized using a wire mesh screen and collected by washing the screen with incomplete MGFL-15. The suspension was then allowed to sit for 5 minutes at room temperature to allow debris to settle and the cells were collected into a new conical tube. Cells were centrifuged at 311 xg for 10 minutes at 4 °C and supernatant removed. The pellets were resuspended in 1.5 mL ACK lysis buffer (Gibco) and incubated at room temperature for 3 minutes. Ten mL

incomplete MGFL-15 was then added and cells were centrifuged at 311 xg for 10 minutes at 4 °C. Cells were then resuspended in incomplete MGFL-15, placed on ice, counted using a haemocytometer, and used for various assays.

### 2.4.4 Isolation of peritoneal leucocytes

Isolation of goldfish peritoneal leucocytes was performed by peritoneal lavage. Goldfish were anesthetized with TMS and sacrificed by cervical dislocation. An incision was made tracing around the left pectoral fin to create a window. Ten mL of ice-cold 1X $PBS^{-/-}$ was injected into fish, 1 scale above the mid-line, approximately 2/3 of the length of the fish using a hooked 18-gauge needle. Lavage fluid was collected from the peritoneal window in a 50 mL conical tube. The contents of the tube were immediately placed on ice until further use.

### 2.4.5 Isolation of mononuclear cells

Leucocytes were isolated as described above, and resuspended in incomplete MGFL-15. Cells were then layered over a 51% Percoll solution (51 mL Percoll, 10 mL 10X $PBS^{-/-}$, 39 mL incomplete MGFL-15) and centrifuged for 25 minutes at 400 xg at 4 °C. Cells at the 51% Percoll/medium interface were collected and transferred into a new conical tube and washed twice with incomplete MGFL-15 (centrifuged at 311 xg for 10 minutes at 4 °C). Cells were placed in a 5 mL polystyrene round bottom tube (BD Falcon) and mononuclear cells were allowed to adhere for 4 hours. The non-adherent lymphocytes were then removed by decanting the supernatant. Isolation of mononuclear cells was confirmed by Hema3 staining.

### 2.4.6 Isolation of neutrophils

Leucocytes were isolated as described above and resuspended in incomplete MGFL-15. Cells were then layered over a 51% Percoll and centrifuged for 25 minutes at 400 xg at 4 °C. All the liquid was decanted, leaving behind the erythrocyte/neutrophil pellet. The pellet was resuspended using ACK lysis buffer as described above to lyse erythrocytes. Neutrophils were then washed twice with 10 mL of incomplete MGFL-15.

### 2.4.7 Isolation of supernatants

Peritoneal lavage fluid was centrifuged at 411 xg for 10 minutes at 4 °C. Supernatants were then immediately placed in a new conical tube and either used immediately or prepared for analysis by Western blotting. When supernatants were being analysed by Western blot, samples were diluted 1:1 in non-reducing 2X Laemmli buffer (Table 2.5) and boiled for 5 minutes. Samples were then stored at -20 °C with protease inhibitor until further use. When fish were subject to peritoneal lavage and samples were going to be used for protein analysis by Western blot commercially purchased ovalbumin (Sigma) was added to 1X $PBS^{-/-}$ prior to use in peritoneal lavage at a concentration of 10 $mg \bullet L^{-1}$. This was for subsequent use as a loading control when cells were removed and supernatants analysed.

## 2.5 Cell bioassays

### 2.5.1 Respiratory burst assay

Cells were harvested following activation by peritoneal lavage at the indicated time point and placed into 5 mL polystyrene round bottom tubes (BD Falcon). Cells were resuspended in 1X $PBS^{-/-}$. Dihydrorhodamine (DHR, Molecular Probes) was added to

cells at a final concentration of 10 μM and incubated for 5 minutes at 20 °C to allow cells to uptake DHR. Phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich) was then added at a final concentration of 100 ng•mL$^{-1}$. Cells were incubated for an additional 30 minutes at 20 °C to allow oxidation of DHR. All samples were staggered with respect to timing to accommodate for the transient state of oxidized DHR fluorescence. Proportion of fluorescent cells was determined using a flow cytometer (BD FACSCanto II). All flow cytometric profiles were analysed using the gating strategy as outlined in Figure 2.2.

### 2.5.2 Nitric oxide assay

Cells were harvested following activation by peritoneal lavage at the indicated time point and placed into 5 mL polystyrene round bottom tubes. Cells were resuspended in 1X PBS$^{-/-}$. 4-Amine-5-Methylamino-2'7'-Difluorofluorowscein Diacetate (DAF-FM Diacetate, Molecular Probes) was added to the cells at a final concentration of 1 μM and incubated for 45 minutes at 20 °C to allow DAF-FM diactate to enter the cells and be converted, by intracellular esterases, to the membrane impermeable DAF-FM. Cells were then washed with 1 mL of 1X PBS$^{-/-}$ to remove any excess DAF-FM diacetate and the incubated for an additional 30 minutes at 20 °C to allow complete de-esterification of the intracellular diacetates. Intracellular DAF-FM binds to nitric oxide, forming a fluorescent benzotriazole derivative. All samples were staggered with respect to timing to accommodate for the transient state of fluorescence. Proportion of fluorescent cells was determined using a flow cytometer (BD FACSCanto II). All flow cytometric profiles were analysed using the gating strategy as outlined in Figure 2.3

### 2.5.3. Cellular infiltration

Cells were harvested following activation by peritoneal lavage at the indicated time point. Cells were counted using a haemocytometer. Within these time points changes in cellular numbers were largely associated with cellular infiltration.

### 2.5.4 Apoptotic cells

Primary kidney leucocytes were isolated a described above (2.4.3). Total leucocytes were then suspended in MGFL-15 supplemented with 10% heat-inactivated fetal calf serum (Gibco), 5% heat-inactivated carp serum, 100 $U \bullet mL^{-1}$ penicillin, 100 $U \bullet mL^{-1}$ streptomycin, and 100 $\mu g \bullet mL^{-1}$ gentamicin. Apoptotic cells were generated by incubating cells for 24 hours at 20 °C in the presence of 10 $\mu g \bullet mL^{-1}$ cyclohexamide. Apoptotic cells were then washed twice in 1X $PBS^{-/-}$ (311 xg for 10 minutes at 4 °C).

## 2.6 Detection of transferrin cleaving activity

### 2.6.1 Detection of transferrin cleavage ability

Detection of transferrin cleaving activity was performed by incubating 2.5 $\mu g \bullet mL^{-1}$ of commercially purchased bovine transferrin (Sigma) with various supernatants and cell preparations believed to contain enzymes capable of cleaving transferrin. All incubations with bovine transferrin were performed for 4 hours at 20 °C. Transferrin cleavage was subsequently detected by Western blot analysis using commercially purchased rabbit anti-bovine transferrin (Abexxa) as the primary antibody.

### 2.6.2 Cellular lysis assay

Goldfish leucocyte sub-populations were isolated as described above. To detect

transferrin cleavage ability of leucocyte sub-populations 1.0 x $10^6$ cells were placed into 5 mL polystyrene round bottom tubes, centrifuged at 311 xg for 10 minutes at 4 °C and all liquid gently removed by aspiration. MilliQ water was then added for 1 minute to lyse all cells, and then the lysed suspension was equilibrated with an appropriate volume of 10X PBS $^{-/-}$. The cellular preparation was then incubated with bovine transferrin in a total volume of 500 µL in 5 mL polystyrene round bottom tubes. After incubation the entire preparation was immediately diluted 1:1 in non-reducing 2X Laemmli buffer and boiled for 10 minutes. Samples were then stored at -20 °C with protease inhibitor until further use.

### 2.6.3 Soluble enzyme cleavage of bovine transferrin

Goldfish serum was isolated as described earlier. Peritoneal lavage supernatants were isolated as described earlier, however 3 mL of ice-cold 1X PBS$^{-/-}$ was used instead of 10 mL. To detect transferrin cleavage ability of goldfish serum was incubated with purified bovine transferrin. Goldfish serum was diluted 1:4 with 1X PBS$^{-/-}$ in a final volume of 500 µL in 5 mL polystyrene bound bottom tubes. To detect the transferrin cleavage ability of peritoneal lavage supernatants bovine transferrin was incubated in 500 µL of peritoneal lavage supernatant in 5 mL polystyrene round bottom tubes. After incubation the entire preparation was immediately diluted 1:1 in non-reducing 2X Laemmli buffer and boiled for 5 minutes. Samples were then stored at -20 °C with protease inhibitor until further use.

### 2.6.4 Leucocyte processing of bovine transferrin cleavage products

Bovine transferrin cleavage products were generated by incubating peritoneal lavage supernants, isolated from fish 18 hours post-zymosan injection, with 2.5 µg•mL$^{-1}$

of commercially purchased bovine transferrin *ex vivo* for 4 hours at 20 °C in 5 mL polystyrene round bottom tubes. Inflammatory leucocytes were isolated by peritoneal lavage 18 hours post-zymosan injection and one million ($1 \times 10^6$) inflammatory leucocytes were incubated with the cleaved bovine transferrin preparation for the indicated time at 20 °C. The suspension was then centrifuged at 400 x g for 10 minutes and the supernatants collected, diluted 1:1 in non-reducing 2X Laemmli buffer, boiled for 5 minutes, and then stored at -20 °C with protease inhibitor until further use.

### 2.6.5 Enzyme cleavage of bovine transferrin

To demonstrate that transferrin cleavage was facilitated by enzymes, opposed to another bioactive mediator, bovine transferrin was incubated with various preparations containing enzymes and then analysed by Western blot to detect transferrin cleavage products. The various preparations were: (1) 50 $ng \bullet mL^{-1}$ commercially purchased porcine elastase IV (Sigma) incubated with 2.5 $\mu g \bullet mL^{-1}$ bovine transferrin (1:50 enzyme/protein mix) at 37 °C for 4 hours; (2) one million ($1.0 \times 10^6$) inflammatory leucocytes isolated 18 hours post zymosan injection, lysed as previously described (*2.6.2*), and incubated with bovine transferrin at 18 °C for 4 hours; (3) peritoneal supernatants isolated 18 hours post zymosan injection and incubated with bovine transferrin at 18 °C for 4 hours; and as a negative control (4) sterile 1X $PBS^{-/-}$ incubated with bovine transferrin ) at 37 °C for 4 hours. Two parallel preparations were also analysed where a) boiled for 5 minutes prior to the introduction of bovine transferrin to denature the enzymes and b) samples were incubated with protease inhibitors.

### 2.6.6 Detection of *in vivo* transferrin cleavage products

Supernatants from peritoneal lavage was isolated as described earlier. To detect *in*

*vivo* goldfish transferrin cleavage products samples were analysed by Western blot using rabbit anti-carp transferrin polyclonal antibody (9AG7), kindly donated by Dr. Miodrag Belosevic. Peritoneal lavage supernatants were collected at the indicated time point after peritoneal injection with the indicated stimulus, immediately diluted 1:1 in non-reducing 2X Laemmli buffer, and boiled for 5 minutes. Samples were then stored at -20 °C with protease inhibitor until further use.

## 2.7 semi-Quantitative PCR

### 2.7.1 RNA isolation

RNA was isolated from peritoneal lavage cells using a Qiagen RNeasy kit (Qiagen) according to manufactures specifications. Briefly, cells were lysed in Buffer RLT. One volume of 70% ethanol was added to the lysate and applied to the spin column. Columns were centrifuged at 8000 xg for 15 seconds at room temperature and washed with Buffer RW1. Columns were centrifuged at 8000 xg for 15 seconds at room temperature. Columns were then washed with Buffer RPE and centrifuged at 8000 xg for 2 minutes at room temperature. The column was then placed in a new 1.5 mL microcentrifuge tube and 50 μL of nuclease-free water (Ambion) was added. To elute RNA, columns were centrifuged at 8000 xg for 1 minute at room temperature. The nucleic acid concentration was quantified using a Nanodrop apparatus at an absorbance of 260 nm. Samples were also read at absorbance's of 230 nm and 280 nm to determine phenolic and protein contamination.

### 2.7.2 cDNA synthesis

cDNA synthesis was performed using SMARTScribe Reverse Transcriptase (Clontech) according to the manufactures protocol using poly-dT. Total RNA was used

for first-strand cDNA synthesis using SMARTScribe Reverse Transcriptase (Clontech). RNA was incubated with 0.5 µL of oligo (dT) primer and 0.5 µL of 3' CDS primer (Table 2.6) at 72 °C for 3 minutes. Then the following was combined in a master mix and added to the reaction mixture such that each reaction contained: 2 µL 5X first strand buffer (Clontech), 1 µL of 10 µM dNTPs, 1 µL 20 mM dithiothreitol (Clontech), and 1 µL of SMARTScribe-RT (100 $U \bullet \mu L^{-1}$). The contents were gently mixed and then incubated at 42 °C for 60 minutes. Second strand synthesis was performed by adding 90 µL of a master mix such that each reaction contained the following: 10 µL 10X PCR buffer (1 mM TrisHCl, pH 8.3, 500 mM KCl, 15 mM $MgCl_2$, 0.01% w/v gelatin), 72.6 µL nuclease-free water, 5 µL 5' PCR primer (Table 2.6), 1.6 µL 25 mM dNTP's, and 0.8 µL Taq/PFU DNA polymerase mix (100:1). The reaction was then incubated as follows: 95 °C for 30 seconds, 50 °C for 30 seconds, 72 °C for 15 minutes.

### 2.7.3 RT-PCR conditions

RT-PCR was performed to determine the relative abundance of transcripts present in peritoneal leucocytes. Primers used amplify mRNA transcripts were ordered from Integrated DNA Technologies (Table 2.7). Target mRNA transcripts were amplified by combining the following reagents in order into a master mix such that each reaction contained the following: 40.3 µL nuclease-free water, 5 µL 10X PCR buffer, 0.8 µL dNTPs (25 mM), 1.2 µL of forward and reverse primers (25 mM), and 0.5 µL DNA polymerase (5U/µL). The master mix was gently mixed and then 49 µL was added to each tube. Finally, 1 µL of the appropriate cDNA template was added and samples immediately placed in a thermocycler using the following parameters: (1) 94 °C, 3

minutes; (2) 94 °C, 1 minute; (3) 60 °C, 1 minute; (4) 72 °C, 1 minute and 30 seconds; 28 cycles of step (2) – (4); (5) 72 °C, 10 minutes. Bands were visualized by separation on a 1% agarose gel. Gels were stained with an ethidium bromide solution for 20 minutes, followed by destaining in distilled water for 10 minutes. Gel images were acquired using an AlphaImager 2200 (Alpha Innotech).

## 2.8 SDS-PAGE electrophoresis and Western blot analysis

### 2.8.1 SDS-PAGE electrophoresis

Samples were analysed by non-reducing SDS-PAGE using the methods originally described by Laemmli (Laemmli, 1970). Samples were electrophoresed through 5% stacked, 10% continuous separating polyacrylamide gels at 110 V for 1 hour and 45 minutes. Electrophoresis was carried out in SDS-running buffer (Table 2.8). Proteins were then transferred onto 0.2 μm nitrocellulose membranes at 100 V for 60 minutes in transfer buffer (Table 2.9).

### 2.8.2. Western blot analysis

After protein transfer, nitrocellulose sheets were washed once with Tween 20/Tris-buffered saline TBS-T (Table 2.10) and then placed in blocking buffer (5% skim milk powder (w/v) (BD Difco) in TBS-T). Nitrocellulose sheets were incubated in blocking buffer on a rocking platform for 60 minutes at room temperature. Nitrocellulose sheets were cut along the 50-kDa molecular weight marker and incubated separately in primary antibody. The appropriate primary antibody was then added to the blocking buffer at the following dilutions to detect full-length, and cleaved transferrin products: (1) full-length bovine transferrin; $1 \bullet 1000^{-1}$, (2) cleaved bovine transferrin; $1 \bullet 750^{-1}$; (3) full-length goldfish transferrin; $1 \bullet 10\ 000^{-1}$; (4) cleaved goldfish transferrin; $1 \bullet 750^{-1}$. The

nitrocellulose sheets were incubated on a rocking platform overnight at 4 °C. Protein bands were visualized using a horseradish peroxidase conjugated goat anti-rabbit IgG (H + L) secondary antibody (JacksonImmunoResearch). Briefly, the primary antibody was removed by extensive washing with TBS-T. The secondary HRP-conjugated antibody was added to the nitrocellulose sheets in blocking buffer at a $1 \bullet 25\ 000^{-1}$ dilution. The nitrocellulose sheet was incubated for 60 minutes at room temperature on a rocking platform. Secondary antibody was removed by extensive washing with TBS-T. The bands were visualized using the WesternBright ECL substrate (Advansta), according to manufactures protocol.

Nitrocellulose sheets were then washed twice in TBS-T and then incubated with stripping buffer (200 mM glycine, 0.1% (w/v) SDS, 0.1% Tween 20, pH 2.2) for 10 minutes on a rocking platform at room temperature. Nitrocellulose sheets were then extensively washed with TBS-T, incubated in blocking buffer for 60 minutes at room temperature, and incubated with mouse anti-ovalbumin monoclonal antibody (Santa Cruz) at a $1 \bullet 1000^{-1}$ dilution on a rocking platform overnight at 4 °C. Protein bands were visualized using a horseradish peroxidase conjugated goat anti-mouse IgG (H + L) (R&D Systems) secondary antibody at a dilution of $1 \bullet 25\ 000^{-1}$ and WesternBright ECL substrate as previously described. Membranes were imaged with film (Kodak).

### 2.8.3. Optimization of Western blot conditions

#### 2.8.3.1 Reducing vs. non-reducing conditions

To determine if samples could be analysed in reducing conditions, identical samples were prepared: a $1 \bullet 2000^{-1}$ dilution of goldfish serum, peritoneal lavage fluid from a non-injected goldfish, and peritoneal lavage fluid from a fish injected with

zymosan. Samples were then diluted 1:1 in either non-reducing 2X Laemlli buffer, or reducing 2X Laemmli buffer (Table 2.6). Samples were then subject to SDS-PAGE and Western blotting as described above (Figure 2.4).

#### 2.8.3.2. Preparation of a positive control of transferrin cleavage products

To generate a positive control for goldfish transferrin cleavage products for use in Western blot increasing concentrations of neutrophil lysate, isolated by peritoneal lavage and lysed as described earlier, was incubated with 10% goldfish serum for 4 hours at 20 °C. Isolation of neutrophils was confirmed by FACS plot, Hema3 staining, and Sudan black staining as described above (Figure 2.5). Samples were then subject to SDS-PAGE and Western blotting as described above (Figure 2.6).

#### 2.8.3.3. Preparation of an exposure control when samples were separated across multiple gels

When samples that were run on separate gels were being compared a positive exposure control was prepared, that consisted of a master mix of diluted goldfish serum. This master mix would be loaded into two lanes of each gel, at either end of the gel (Figure 2.7).

## 2.9 Analysis

All flow cytometry data was collected on a FACSCanto II flow cytometer (BD Biosciences) and data was analysed using FCS Express software. Statistics were performed using Prism 4 software (GraphPad Prism). Densitometry analysis for RT-PCR was performed by uploading images to ImageJ and relative band intensity was determined for each band relative to the β-actin control.

**Table 2.1 – Composition of 10X PBS$^{-/-}$.**

| Component | Amount (g) |
|---|---|
| KCl | 2.00 |
| $KH_2PO_4$ | 2.00 |
| NaCl | 80.00 |
| $Na_2HPO_4$•7H2O | 21.60 |
| MilliQ water (18.2Ω) | Top to up 1 L |

**Table 2.2 – Composition of incomplete MGFL-15 media.**

| 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 (18.2Ω) | Top up to 2 L |

*See table 2-3 for composition
*See table 2-4 for composition
***GFL-15 is made by mixing DMEM and Leibovitz-15 media powders in 2 L of MilliQ.

**Table 2.3 – Composition of nucleic acid precursor solution.**

| Component | Amount (g) |
|---|---|
| Adenosine | 0.067 |
| Cytidine | 0.061 |
| Hypoxanthine | 0.03 |
| Thymidine | 0.061 |
| Uridine | 0.061 |
| MilliQ water (18.2Ω) | 100 mL |

**Table 2.4 – Composition of 10X Hank's Balance Salt Solution**

| Component | Amount (g) |
|---|---|
| KCl | 2.00 |
| $KH_2PO_4$ | 0.30 |
| NaCl | 40.00 |
| $Na_2HPO_4$•7H2O | 0.45 |
| D-glucose | 5.00 |
| Phenol red | 0.05 |
| MilliQ water (18.2Ω) | Top up to 500 mL |

**Table 2.5 – Composition of non-reducing 2X Laemmli buffer.**

| Component | Amount |
|---|---|
| Sodium dodecyl sulfate | 0.40 g |
| Glycerol | 2 mL |
| 0.5 M Tris• HCl | 2.5 mL |
| Bromophenol blue | Pinch |
| MilliQ water (18.2Ω) | Top up to 10 mL |

*for reducing 2X Laemmli buffer 1 mL of MilliQ water was substituted with 1 mL β-mercaptoethanol

**Table 2.6 – Primers used in cDNA synthesis**

| Primer | 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 2.7 – Primer sequences used for RT-PCR.**

| Primer | Sequence (5' – 3') |
|---|---|
| iNOS forward | GGA GGT ACG TCT GCG AGG AGG CT |
| iNOS reverse | CCA GCG CTG CAA ACC TAT CAT CCA |
| Transferrin forward | GCT CAT CTC GTT TCT GGC GTG CC |
| Transferrin reverse | GAT CAG CAC CAG GAG CGC AGC C |
| Mx forward | ACA GAA GGA ACT GGA GGC GTA |
| Mx reverse | CGC AGG TTC CTC CAA CAG C |
| β-actin forward | CGA GCT GCG TGT TGC CCC TGA G |
| β-actin reverse | CGG CCG TGG TGG TGA AGC TGT AG |

**Table 2.8 – Composition of SDS Running Buffer**

| Component | Amount (g) |
|---|---|
| Tris | 30.00 g |
| Glycine | 144.00 g |
| Sodium dodecyl sulfate | 10.00 g |
| MilliQ water (18.2Ω) | Top up to 10 L |

**Table 2.9 – Composition of Transfer Buffer**

| Component | Amount |
|---|---|
| Tris | 12.16 g |
| Glycine | 57.68 g |
| Methanol | 800 mL |
| MilliQ water (18.2Ω) | Top to 4 L |

**Table 2.10 – Composition of TBS-T**

| Component | Amount |
|---|---|
| NaCl | 29.22 g |
| 1M Tris• HCl | 40 mL |
| 10 % Tween-20 | 40 mL |
| MilliQ water (18.2Ω) | Top up to 4L |

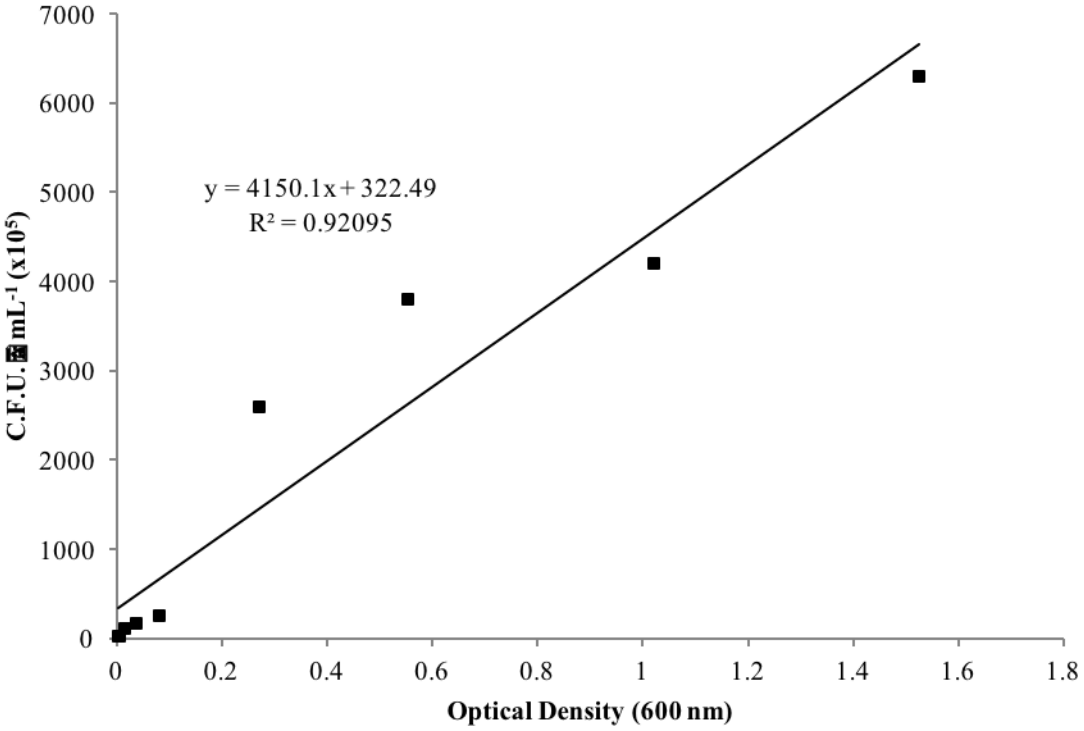



**Figure 2.1 – *Aeromonas veronii* growth curve.** *A. veronii* was grown at room temperature for 8 hours. Every hour a 1 mL aliquot was removed. Optical density (OD) and colony forming units (CFU) were determined for each time point.

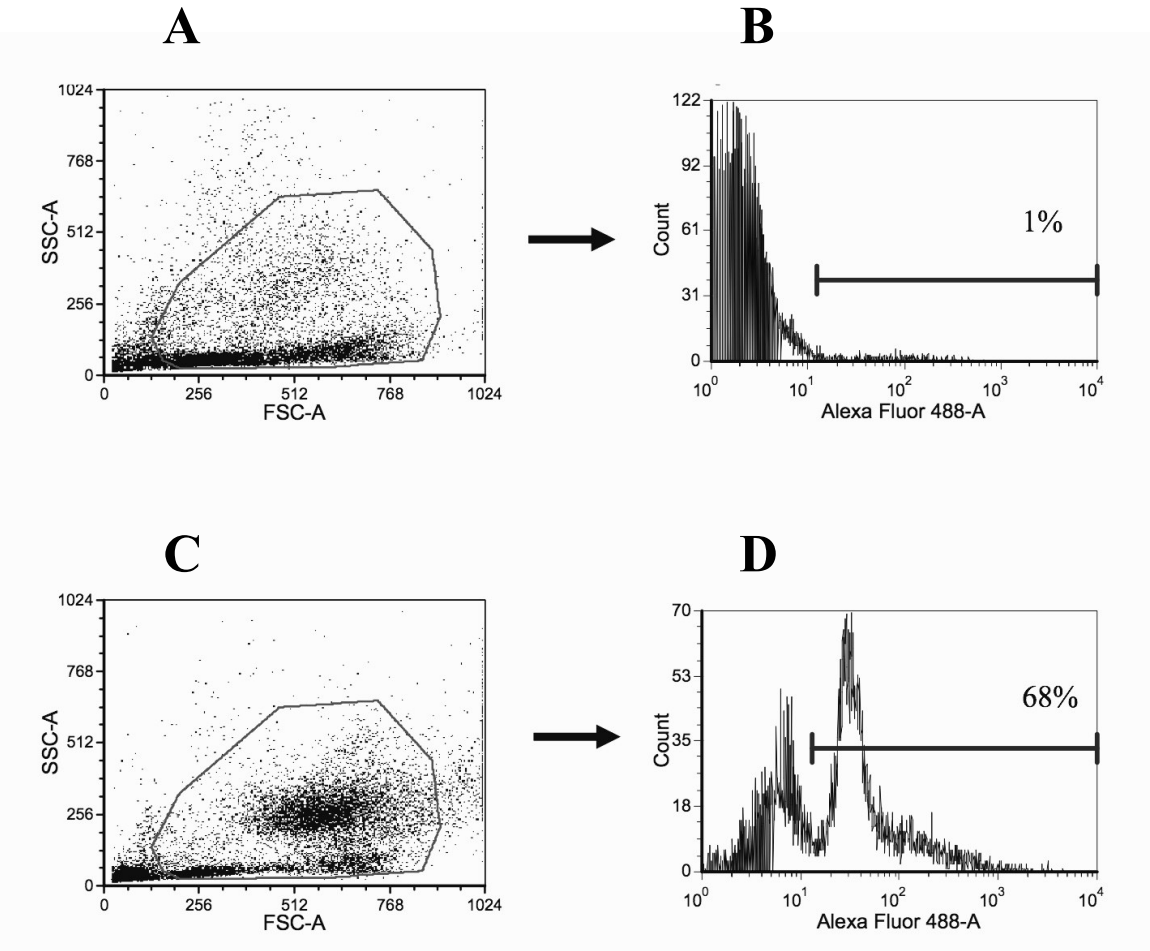
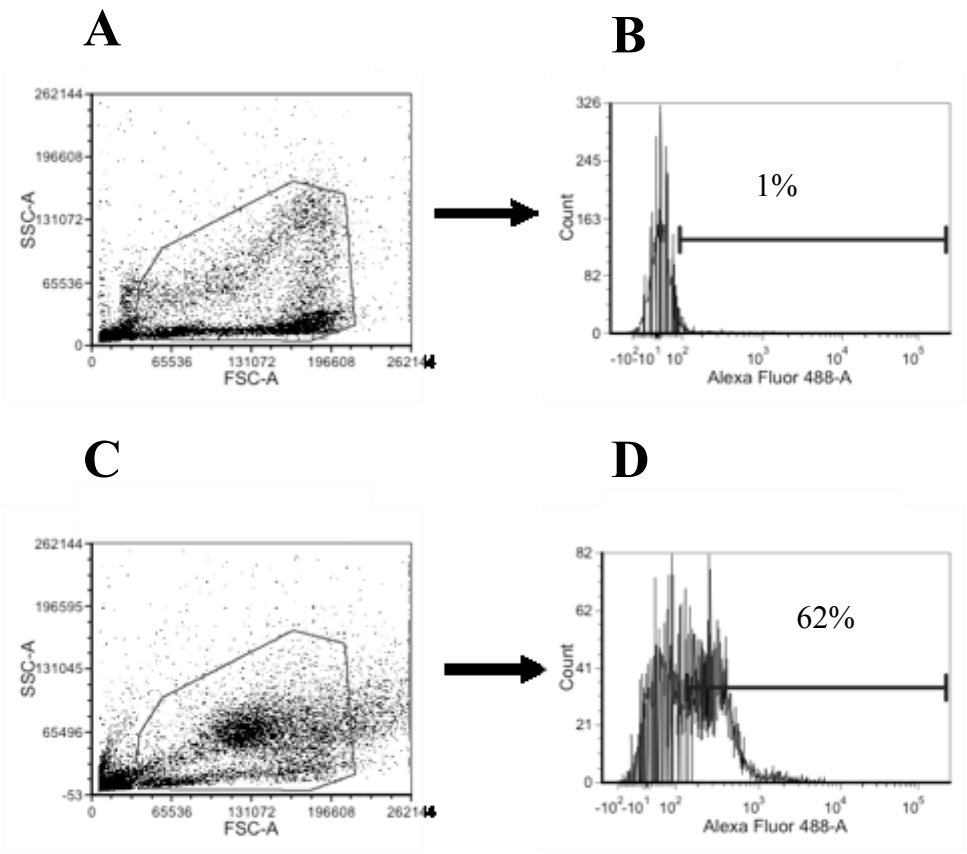



**Figure 2.2 – DHR gating strategy for ROS production.** Samples were collected on a BD FACSCanto II flow cytometer and analysed using FCS express. (A) Scatter plot depicting the relative internal complexity (SSC-A) and size (FSC-A) of leucocytes isolated by peritoneal lavage from non-injected goldfish. (B) Histogram depicting the relative Alexa-floura 488 fluorescence positivity from leucocytes gated in (A). (C) Scatter plot depicting the relative internal complexity (SSC-A) and size (FSC-A) of leucocytes isolated by peritoneal lavage 18 hours post-zymosan injection (D) Histogram depicting the proportion of leucocytes considered positive for DHR staining from those gated in (C).




were
. (A)
Scatter plot depicting the relative internal complexity (SSC-A) and size (FSC-A) of leucocytes isolated by peritoneal lavage from non-injected goldfish. (B) Histogram depicting the relative Alexa-floura 488 fluorescence positivity from leucocytes gated in (A). (C) Scatter plot depicting the relative internal complexity (SSC-A) and size (FSC-A) of leucocytes isolated by peritoneal lavage 24 hours post-zymosan injection (D) Histogram depicting the proportion of leucocytes considered positive for DAF-FM diacetate staining from those gated in (C).

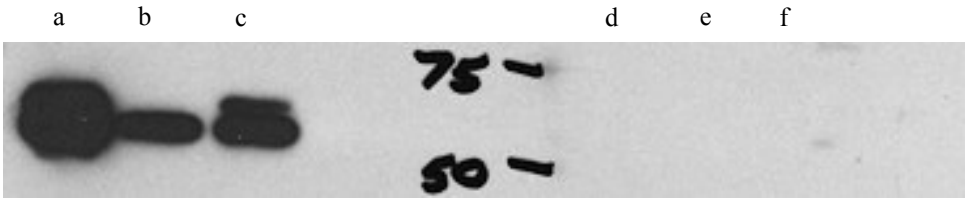



**Figure 2.4 – Reducing v. non-reducing.** Under reducing conditions 9AG7 is unable to detect transferrin. Two sets of three samples were prepared for Western blotting in the absence (a-c) or presence (d-f) of β-mercaptoethanol**.** The three samples were (a/d) – 1:2000 dilution of goldfish, *C. auratus,* serum, (b/e) – Peritoneal lavage fluid from non-injected fish, (c/f) – Peritoneal lavage from zymosan injected fish.

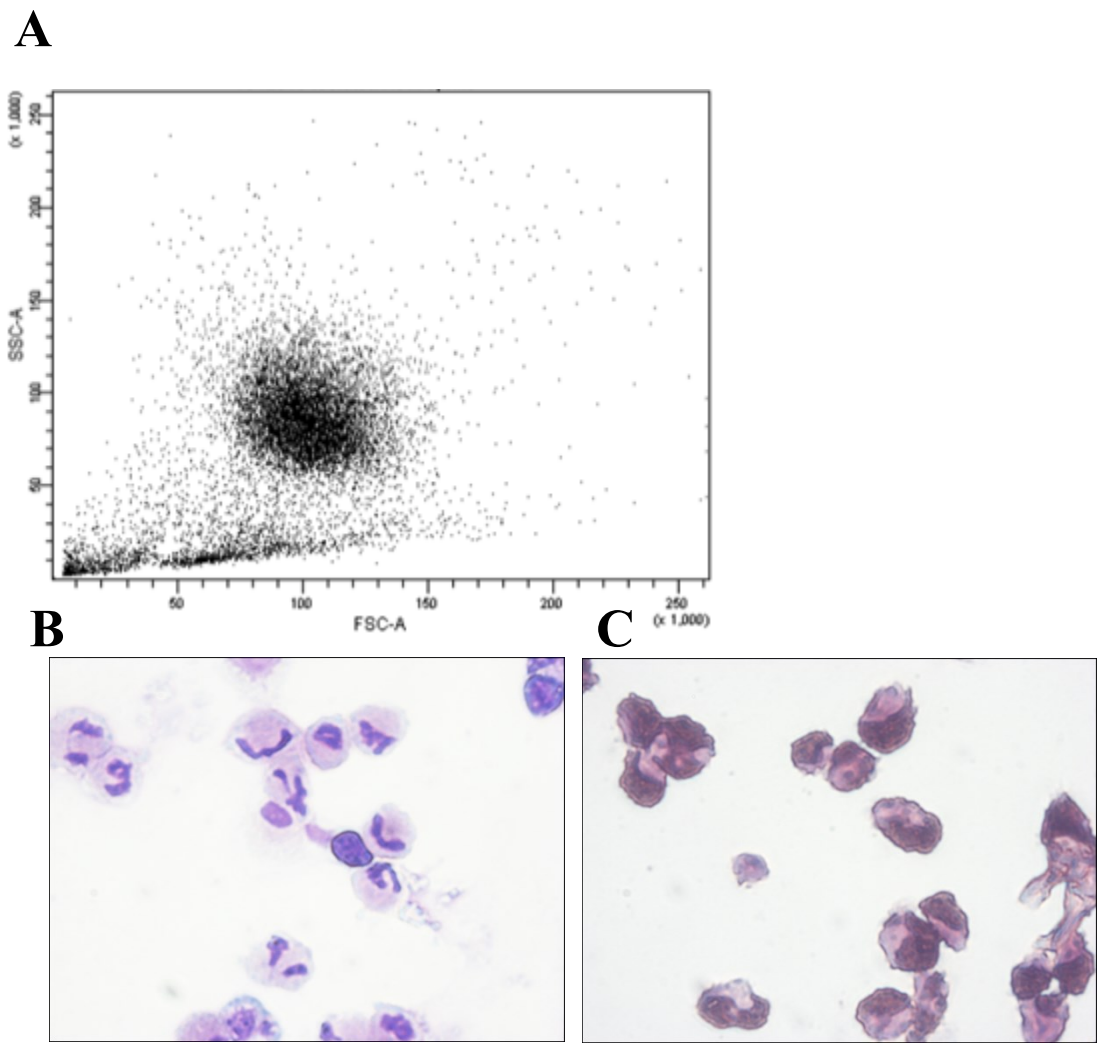



**Figure 2.5 – Isolation of neutrophils.** Goldfish, *C. auratus,* were injected intraperitoneally with 2.5 mg of zymosan and non-adherent cells were harvested 18 hours post injection. Erythrocytes were lysed and neutrophils were isolated using a percoll gradient. Neutrophils were evaluated using (A) FACS plot. (B) Modified Wright-Giesma stain. (C) Sudan black stain.

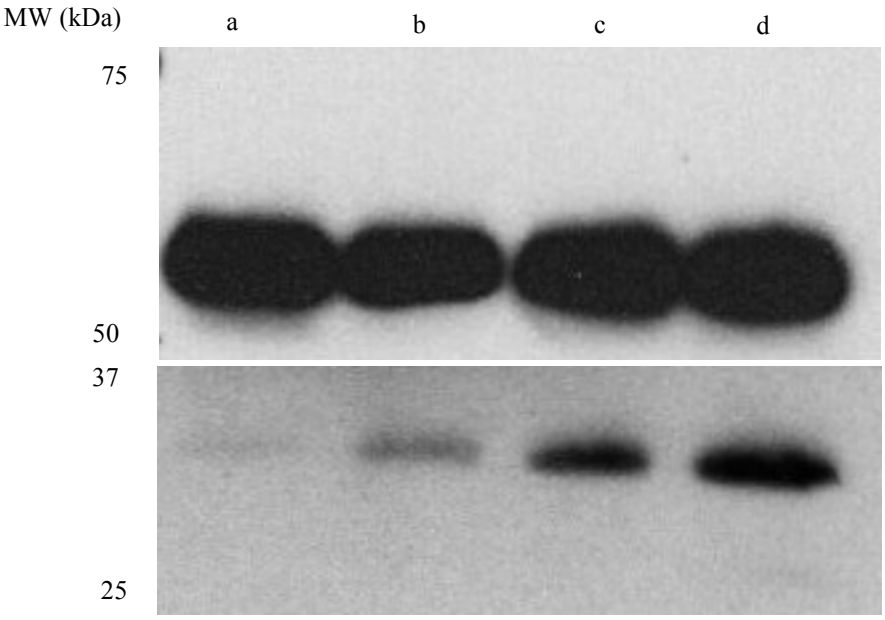



**Figure 2.6 – Goldfish transferrin is cleaved by neutrophil lysate in a dose-dependent manner.** Increasing concentrations of neutrophil lysate was incubated in the presence of 10% goldfish serum for 4 hours at 18 °C. Samples were then subject to Western blotting to detect the presence of native and cleaved transferrin. The concentration of neutrophil lysate used was (a) 250 000 cells. (b) 500 000 cells. (c) 1 000 000 cells. (d) 2 000 000 cells.

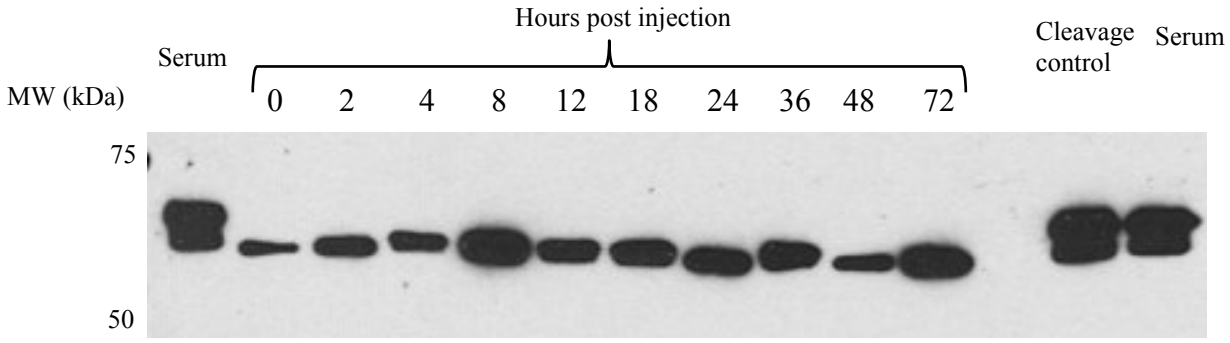



**Figure 2.7 – Exposure control when samples were run across multiple gels**. When time-course samples were run over multiple gels a master mix of serum was loaded in all gels to be able to normalize the amount of native form present in Western blots.

# Chapter 3: Cleaved transferrin as an early marker of acute inflammation in goldfish, *Carassius auratus*

## 3.1 Introduction

The historical role of transferrin is as an iron transport protein, delivering iron to cells. However transferrin is an evolutionary conserved protein, with homologues in organisms as distant as marine-algae, that has been shown to have many other biological functions (Lambert, 2012). Transferrin has been shown to play a role in maturation of leucocytes (Evans et al., 1989; Macedo et al., 2004), sequestering iron from invading pathogens (Ellis, 2001; Skaar, 2010), and is a highly conserved acute phase protein (Bayne and Gerwick, 2001; Cray et al., 2009; Xie et al., 2002a; Yoshiga et al., 1997). Transferrin also participates directly in bacterial killing, preventing bacterial growth (Ibrahim et al., 1998; Ibrahim et al., 2000; van der Kraan et al., 2004; Yun et al., 2009). *In vitro* endogenous cleavage of transferrin can activate teleost and murine macrophages (Haddad and Belosevic, 2009; Stafford and Belosevic, 2003; Stafford et al., 2001) while full-length ovotransferrin can activate avian macrophages and granulocytes (Xie et al., 2002b). Indeed, transferrin is a multifunctional immune molecule capable of initiating early activation events during immune responses, but also participating in the effector responses against pathogens.

Although cleaved transferrin has been shown to activate macrophages *in vitro* the presence of endogenously produced cleaved transferrin products has not been shown *in vivo*. Given the plethora of functions attributed to transferrin during the initiation and effector stages during immune responses cleaved transferrin may have a significant role during the early initial activation events of acute inflammation. Acute inflammation is a tightly regulated, highly conserved process, in response to tissue damage or pathogen

invasion. Acute inflammation is typically characterized by rapid recruitment of macrophages and neutrophils to the inflammatory site, subsequent production of soluble mediators and induction of anti-microbial responses (Serhan and Savill, 2005). Current markers of acute inflammation have focused on these early events, specifically the production of pro-inflammatory cytokines such as TNF-α, IL-1β, and IFN-γ. These current markers of acute inflammation are typically synthesized and utilized rapidly, and are present in small quantities (Cui et al., 2000). Given that transferrin, and transferrin-like molecules in addition to macrophages, and macrophage-like cells are found in almost all organisms I propose that transferrin may be a highly conserved immune molecule, that plays a integral role in the initiation events of acute inflammation in several evolutionary groups. Transferrin is also typically present in high quantities, 2 – 5 $mg \bullet mL^{-1}$ (Regoeczi and Hatton, 1980), making it an excellent candidate to be a good comparative marker of early events during acute inflammation.

In this chapter I will use a self-resolving, non-infectious, model of acute inflammation in goldfish, *Carassius auratus*, to evaluate the presence of cleaved transferrin products *in vivo*. I will also use several well-defined characteristics of the inflammatory site to correlate the appearance of transferrin fragments to the magnitude of acute inflammatory responses. Specifically I will evaluate the magnitude of leucocyte infiltration, respiratory burst, and NO responses. The main objectives of this chapter were to (1) demonstrate the presence of endogenous production of cleaved transferrin *in vivo* and (2) assess the ability of cleaved transferrin products to serve as a comparative marker of acute inflammation with differential inflammatory stimuli.

## 3.2 Results

### 3.2.1 Intraperitoneal injection of zymosan induces robust anti-microbial responses

In mammals, intraperitoneal administration of zymosan induces a robust inflammatory response, characterized by rapid neutrophil influx and synthesis of pro-inflammatory prostaglandins (Doherty et al., 1985). This self-resolving model of acute inflammation has also been adapted and well characterized for use in goldfish previously (Havixbeck et al., 2016; Rieger et al., 2015; Rieger et al., 2012). To characterize the microenvironment at the inflammatory site and help separate the induction, effector, and resolution phases of acute inflammation I evaluated the magnitude of leucocyte infiltration, respiratory burst, and NO production (Figure 3.1). Similar to previous work I observed that leucocyte infiltration (Figure 3.1A) and respiratory burst (Figure 3.1B) reached maxima 18 hours post injection while NO production, or accumulation, by leucocytes reached a maximum 24 hours post injection (Figure 3.1C). All parameters returned to homeostatic levels by 72 hours post injection (Figure 3).

### 3.2.2 Zymosan peritonitis induces expression of iNOS

*In vitro* cell culture of teleost macrophages has shown an increase in iNOS expression as early as 2 hours post stimulation (Stafford and Belosevic, 2003). I aimed to investigate the gene expression of iNOS by peritoneal leucocytes at the inflammatory site to determine if *de novo* iNOS synthesis correlated with the production of NO. RT-PCR and iNOS specific primers were used to evaluate iNOS gene expression by peritoneal leucocytes *in vivo*. Intraperitoneal administration of zymosan induced iNOS expression as early as 4 hours post-injection in all treatment groups (Figure 3.2). Maximum iNOS

expression peaks in all groups by 24 hours post challenge, returning to homeostatic levels by 72 hours post challenge.

### 3.2.3 Zymosan peritonitis induces endogenous cleavage of transferrin *in vivo*

Endogenous, enzyme mediated, production of transferrin cleavage products in teleost systems has been previously shown *in vitro* (Jurecka et al., 2009; Stafford et al., 2001). I used Western blotting with a rabbit anti-carp transferrin primary antibody, 9AG7, to evaluate the presence of cleaved transferrin products at the inflammatory site, along with the banding patterns and abundance of full-length transferrin present in the serum and the inflammatory site (Figure 3.3). I show for the first time endogenously produced transferrin cleavage products *in vivo* (Figure 3.3C). These cleavage fragments are present only after immune challenge. There is also a large degree of heterogeneity in cleavage product banding patterns. There was no apparent correlation between the amount of full-length transferrin detected and the time after immune challenge (Figure 3.3A, B). This heterogeneity is present both in the serum and the peritoneum. I also noted the detection of the polymorphic variants in the native transferrin molecules noted in previous studies (Yang et al., 2004).

### 3.2.4 Intraperitoneal injection of heat-killed *Aeromonas veronii* induces robust anti-microbial responses

*Aeromonas* species have been shown previously to induce anti-microbial responses in several teleost systems (Antipa and Amend, 1977; Chandran et al., 2002; Rodríguez et al., 2008). To evaluate the potential for differential leucocyte mediated responses compared to the zymosan peritonitis model I evaluated the leucocyte infiltration, respiratory burst, and NO responses of goldfish injected intraperitoneally

with 5.0 x $10^6$ C.F.U. of heat-inactivated *A. veronii.* This revealed a similar microenvironment compared zymosan peritonitis model (Figure 3.1). Leucocyte infiltration and respiratory burst responses reached maxima 18 hours post injection while NO production reached a maximum 24 hours post injection (Figure 3.4). However the magnitude of leucocyte infiltration and respiratory burst were lower than responses observed during zymosan peritonitis. Anti-microbial responses returned to homeostatic levels by 72 hours post injection.

### 3.2.5 Heat-killed *Aeromonas veronii* induces endogenous cleavage of transferrin *in vivo*

In addition to characterizing the microenvironment post-challenge with heat-killed *A. veronii* I aimed to determine if injection of heat-killed *A. veronii* could induce the endogenous cleavage of transferrin to produce immunostimulatory transferrin cleavage products. Indeed, addition of exogenous transferrin has been shown to enhance the ability of macrophages to produce NO when challenge with *Aeromonas* species (Stafford and Belosevic, 2003). I used Western blotting with the primary rabbit anti-carp transferrin polyclonal antibody, 9AG7, to evaluate the presence of transferrin cleavage products at the inflammatory site. Similar to zymosan, injection of heat-inactivated *A. veronii* induced the generation of transferrin cleavage products *in vivo* at the inflammatory site (Figure 3.5). There was no correlation between the amounts of full-length transferrin present and the time post challenge (Figure 3.5A, B). The appearance of cleavage products was present only after administration of *A. veronii* (Figure 3.5C). I also noted a large degree of heterogeneity in the banding pattern of cleavage products.

### 3.2.6 Injection of 200 µg and 400 µg poly(I:C) induces leucocyte anti-microbial responses

To determine an appropriate dose of poly(I:C) to use in intraperitoneal injection time course experiments poly(I:C) was titrated, using 100 µg, 200 µg, and 400 µg in 100 µL of 1X $PBS^{-/-}$. Previous work in rainbow trout has shown differential gene expression of the NADPH oxidase units when stimulated with poly(I:C) when compared to zymosan and LPS stimulation (Boltaña et al., 2009). To monitor successful induction of inflammatory microenvironment Mx gene expression was analysed in addition to leucocyte infiltration, respiratory burst, and NO production 24 hours post challenge (Figure 3.6). Intraperitoneal administration of 200 µg and 400 µg poly(I:C) resulted in significant amounts of leucocyte influx (Figure 3.6A), NO production (Fiureg 3.6C), and Mx gene expression (Figure 3.6d). There was no difference in the amount of respiratory burst with any amount of poly(I:C) used (Figure 3.6B), and there was no significant difference between 200 µg and 400 µg. Therefore 200 µg was chosen for further experiments investigating poly(I:C).

### 3.2.7 Intraperitoneal injection of Poly(I:C) induces anti-microbial responses

To examine how the inflammatory microenvironment may differ upon challenge with an intracellular inflammatory stimulus, poly(I:C), compared to the extracellular inflammatory stimuli, zymosan and heat-inactivated *A. veronii,* used in previous experiments I challenged goldfish with 200 µg of poly(I:C) and evaluated the leucocyte infiltration, respiratory burst, and NO production (Figure 3.7). Leucocyte infiltration reached a maximum 24 hours post challenge (Figure 3.7A), while NO production reached a maximum 12 hours post challenge (Figure 3.7C). Respiratory burst responses remained

at basal levels throughout the time course experiments (Figure 3.7B). The number of leucocytes and the proportion of leucocytes producing or accumulating NO were less than both the zymosan and *A. veronii* model.

### 3.2.8 Poly(I:C) induces endogenous cleavage of transferrin *in vivo*

Finally, I investigated the ability of poly(I:C) to induce the production of transferrin cleavage products post-challenge. I used Western blotting with the primary rabbit anti-carp transferrin polyclonal antibody, 9AG7, to evaluate the presence of endogenously cleaved transferrin products post-challenge with poly(I:C). Western blot analysis of peritoneal lavage supernatants from poly(I:C) injected fish indicated that poly(I:C) also induces endogenous cleavage of full-length transferrin *in vivo* (Figure 3.8). Transferrin cleavage products were present as early as 2 hours post challenge. Similar to zymosan and *A. veronii*, there was no correlation in the amount of full-length transferrin present in the serum, or peritoneum with poly(I:C) challenge (Figure 3.8A, B). There was similar heterogeneity in banding patterns of cleavage products as noted previously (Figure 3.8C).

### 3.2.9 Sterile inflammation does not produce transferrin cleavage products

To determine that transferrin cleavage products were generated in response to pathogenic challenge rather than induction of sterile inflammation with 1X $PBS^{-/-}$ I injected goldfish with sterile saline and examined the presence of transferrin cleavage products in peritoneal lavage fluid using Western blotting with the primary rabbit anti-carp transferrin polyclonal antibody, 9AG7. A positive control of peritoneal lavage fluid isolated a zymosan injected fish was used to determine adequate exposure to demonstrate absence of transferrin cleavage products. Transferrin cleavage products were not detected

during the time points examined (Figure 3.9). There was no correlation with the amount of full-length transferrin present after challenge with saline (Figure 3.9A).

## 3.3 Discussion

Inflammation is a critical process that is required for the clearance of pathogens and restoration of homeostasis. However poor regulation mechanisms of inflammatory control can lead to devastating side effects. Failure to induce acute inflammation can lead to uncontrolled pathogen proliferation, while a loss of control can lead to development of chronic inflammatory diseases (Serhan and Savill, 2005). Activation of resident macrophages is crucial for the effective initiation of acute inflammation and propagation of subsequent events of acute inflammation (Soehnlein and Lindbom, 2010). Traditionally, pathogen associated molecular patters (PAMPs) have been regarded as the primary activators of acute inflammation, binding to pattern recognition receptors (PRRs) on resident leucocytes, that then initiate an immune response (Kawai and Akira, 2010). However in addition to leucocytes being able to discriminate self from non-self molecules, they are able to differentiate between healthy- and damaged-self molecules, danger associated molecular patterns (DAMPs) (Zhang and Mosser, 2008). These endogenous damaged molecules share many similarities to PAMPs, and in some cases also bind to the same PRRs. Recently transferrin fragments have been shown to activate teleost and murine macrophages *in vitro* (Haddad and Belosevic, 2009; Stafford et al., 2001). Transferrin fragments may also contribute to the early activation events of acute inflammation *in vivo* by acting as a DAMP. Given that transferrin, and transferrin-like molecules are found in almost all organisms (Lambert, 2012; Lambert et al., 2005)

cleaved transferrin products could be an excellent comparative marker of the pro-inflammatory phase of acute inflammation.

Current markers of the pro-inflammatory phase of acute inflammation have focused on cytokines, such as TNF-α, IL-1β, and IFN-γ. Although these cytokines are important for inducing down-steam effects on leucocytes to propagate acute inflammation they are short-lived, and exert their effects in small quantities. In mammals, challenge with bacterial stimuli resulted in a maximum amount of TNF-α that decreased 90 minutes post-stimulation. In the same study IL-1β decreased 3 hours post-stimulation (Hesse et al., 1988). In a murine model TNF-α production decreased 1 h after *E. coli* challenge (Cui et al., 2000). In goldfish TNF-α expression has been shown to return to homeostatic levels within 12 hours post zymosan challenge, while expression of both IL-1β and IFN-γ return to basal levels within 24 hours (Havixbeck et al., 2016; Rieger et al., 2015). Cleaved transferrin products were detectable as early as 8 hours post challenge with all pathogen challenges (Table 3.1). In some cases cleavage products were detectable as early as 2 hours post challenge (Table 3.1). This early appearance of cleavage products suggests that resident leucocytes may play a role in early transferrin cleavage events. However these cleavage products may be generated by proteases released into the inflammatory site by the adherent cell population surround the inflammatory site. Given that transferrin cleavage products were not seen in the serum, it is unlikely that an active protease is entering directly from the serum into the inflammatory site. Although the appearance of cleaved transferrin products is seen in all pathogen challenges examined in this study there is significant heterogeneity in the banding patterns, and the kinetics of cleavage product detection. The differential banding

patterns of cleavage products could have a multitude of contributing factors. First, the primary rabbit anti-carp transferrin polyclonal antibody, 9AG7, has differential sensitivity to various transferrin breakdown products, with several smaller break down products not even detectable by Western blot (Haddad and Belosevic, 2009). Second, transferrin is a polymorphic protein with three known isoforms characterized in goldfish (

et al., 2004). The various polymorphic variants likely yield transferrin fragments of different molecular weights. Third, there is a diverse array of extracellular matrix proteases expressed during acute inflammation that may contribute to differential transferrin cleavage. Ultimately, although the primary antibody used, 9AG7, was useful in identifying transferrin cleavage products given the limited repertoire of transferrin break down products it can detect, given the plethora of fragments potentially generated, a more useful technique to identify transferrin cleavage products would be the use of mass spectrometry.

A further complicating factor in detection of cleaved transferrin products is, to date; it is not known what receptor cleaved transferrin products bind. Previous studies have suggested that cleaved transferrin exerts its anti-microbial induction effects through TLR binding, given the similarity in macrophage responses when treated with PAMPs or cleaved transferrin products (Haddad and Belosevic, 2009; Jurecka et al., 2009; Stafford and Belosevic, 2003; Stafford et al., 2004). In examining the anti-microbial mechanisms of leucocytes *in vivo* along with the appearance of transferrin cleavage products it was apparent that the leucocyte responses were more uniform than the appearance of cleaved transferrin products. All of the pathogen mimics, or heat-inactivated pathogens, used stimulated leucocytes through well-characterized PRRs. Specifically, zymosan, a fungal

mimic, primarily stimulates leucocytes through dectin-1 and TLR-2 (Dillon et al., 2006). Heat-inactivated *A. veronii*, a gram-negative bacterium, would primarily stimulate through TLR-4 (Medzhitov, 2001). Finally, poly(I:C), a double stranded RNA molecule stimulates leucocytes through TLR-3 (Alexopoulou et al., 2001). Interestingly, these three inflammatory stimuli resulted in differential kinetics of leucocyte mediated anti-microbial responses. Most notably was the marked increase in respiratory burst responses noted with fungal (Figure 3.1B) and bacterial (Figure 3.4B) challenges, not seen in the viral challenge (Figure 3.7 B). Previous work in my lab has characterized the leucocyte and respiratory kinetics of zymosan peritonitis in goldfish, closely following the results seen in these experiments (Havixbeck et al., 2016; Rieger et al., 2015; Rieger et al., 2012). By investigating the nitric oxide response of inflammatory leucocytes I added an additional anti-microbial response to this well characterized model (Figure 3.1 C). The shift in NO production compared to respiratory burst is likely due to the need for leucocytes to produce iNOS *de novo* (Figure 3.2) (Korhonen et al., 2005). In addition to the need to synthesize iNOS, the primary substrate for NO production, L-arginine, is a by-product of respiratory burst, resulting in a subsequent delay in the accumulation of L-arginine from respiratory burst, and then degradation of L-arginine for NO production (Neumann, 1999).

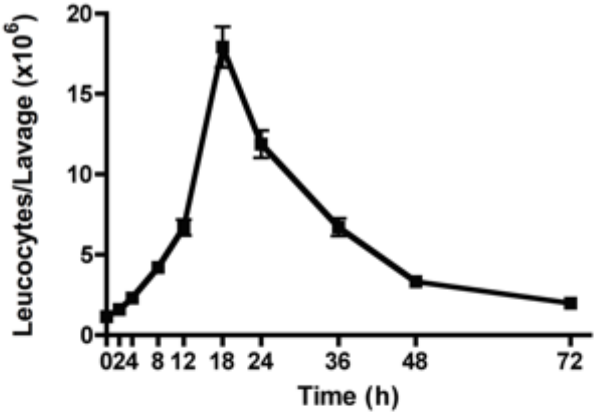
Although the kinetics of cleaved transferrin products detected in these experiments indicate that cleaved transferrin products would not be a reliable marker of acute inflammation, more importantly these experiments reveal the presence of endogenously produced cleaved transferrin products *in vivo*. Given the consistency among anti-microbial leucocyte responses and the heterogeneity of cleaved transferrin

products this suggests that initiation of an immune response is not dependent on transferrin cleavage, however transferrin cleavage may be complimentary, and not redundant, to the induction of acute inflammation *in vivo*.

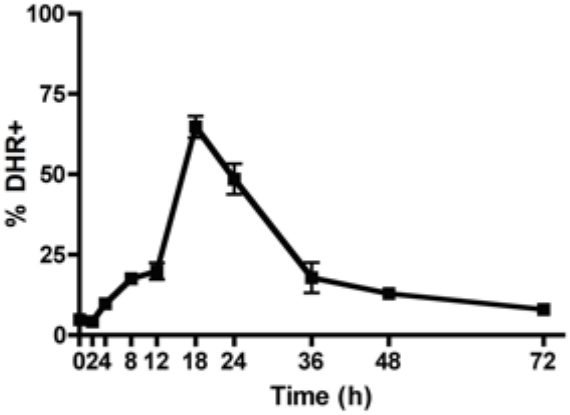
**Table 3.1 – Table of maximum leucocyte mediated responses and transferrin cleavage products during kinetic experiments**

| | Maximum leucocyte infiltration | Maximum respiratory burst | Maximum NO production | 1st appearance of transferrin cleavage products |
|---|---|---|---|---|
| **Zymosan** | 18 hours | 18 hours | 24 hours | 8 hours |
| **Heat-killed *A. veronii*** | 18 hours | 18 hours | 24 hours | 4 – 8 hours |
| **Poly(I:C)** | 24 hours | Not detected | 12 hours | 2 – 4 hours |

**A**


Leucocytes/Lavage (x10^6)
20
15
10
5
0
0 2 4 8 12 18 24 36 48 72
Time (h)

**B**


% DHR+
100
75
50
25
0
0 2 4 8 12 18 24 36 48 72
Time (h)

**C**

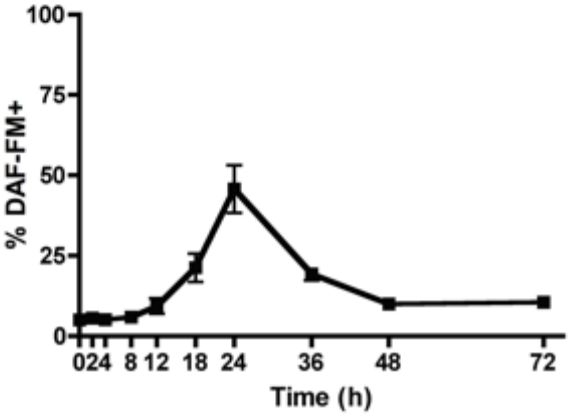

% DAF-FM+
100
75
50
25
0
0 2 4 8 12 18 24 36 48 72
Time (h)

**Figure 3.1 – Zymosan induces anti-microbial responses of goldfish leucocytes.** Goldfish were injected intraperitoneally with 2.5 mg of zymosan (A) Non-adherent leucocytes were isolated by peritoneal lavage and enumerated using a haemocytometer. (B) Respiratory burst in isolated leucocytes was determined using DHR. (C) NO in isolated leucocytes was determined using DAF-FM diacetate. n = 4

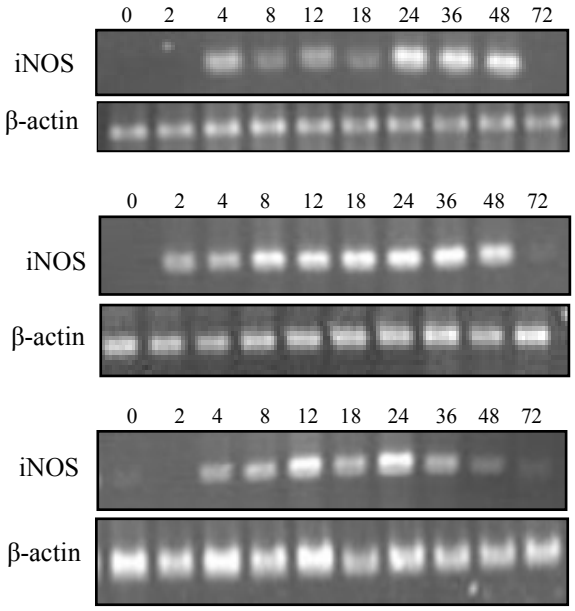



**Figure 3.2 – Zymosan induces increased iNOS expression in goldfish leucocytes.** Goldfish were injected with 2.5 mg of zymosan. Non-adherent leucocytes were harvested by peritoneal lavage and frozen at -80ºC until RNA isolation. RNA was isolated from total cells using a Quiagen RNeasy kit for cells and tissue according to manufacturers instructions. Total RNA was used for cDNA synthesis. cDNA was used as template in PCR reactions to amplify iNOS, expected size 600 b.p., and β-actin, expected size 350 b.p. n = 3. PCR products were amplified with the following conditions (1) 95 ºC; 3 minutes, (2) 95 ºC; 30 seconds, (3) 60 ºC; 30 seconds, (4) 72 ºC; 90 seconds, 28 cycles of (2) – (4), (5) 72 ºC; 10 minutes.

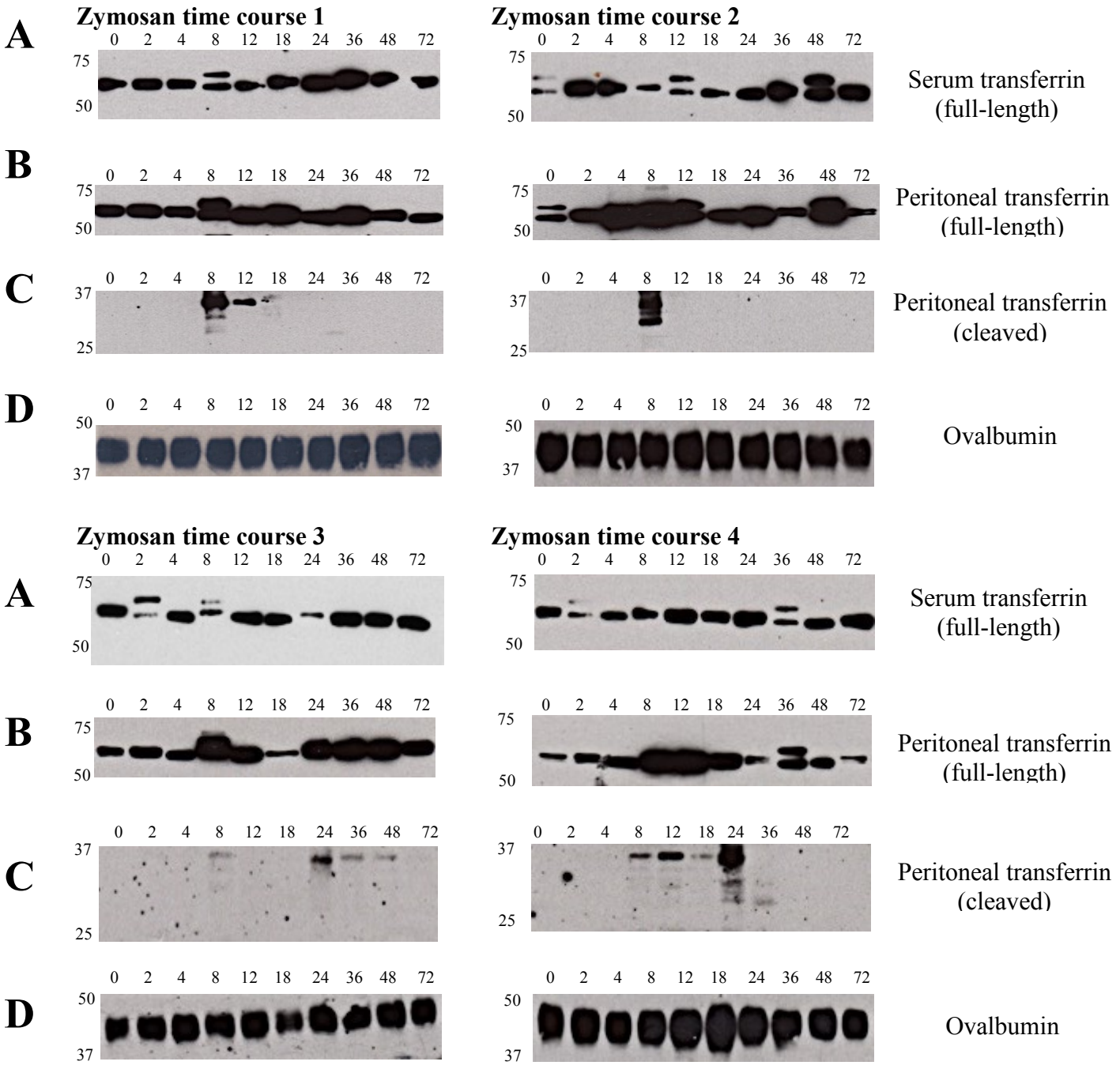
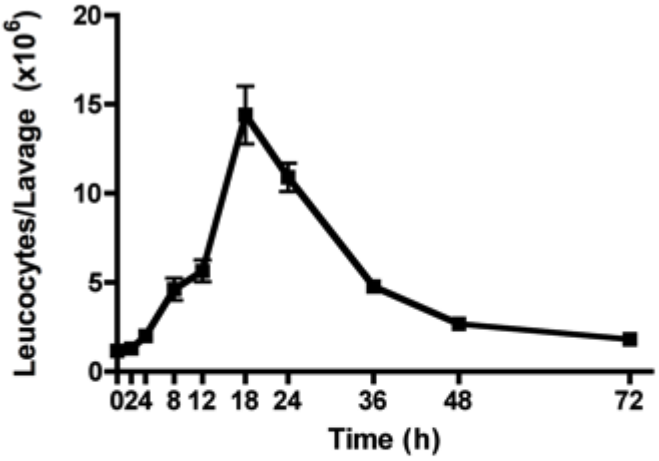
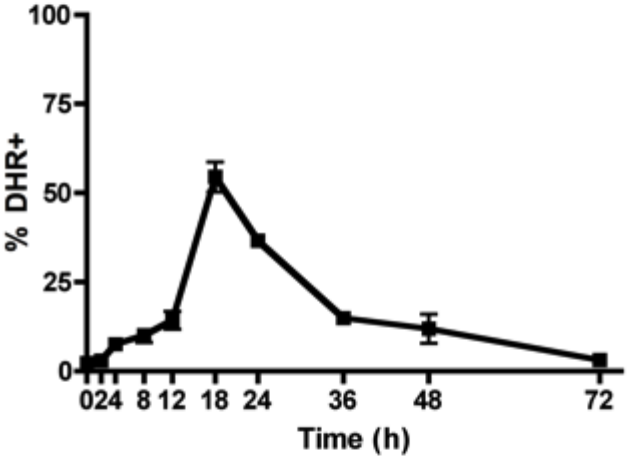

A
Zymosan time course 1
0 2 4 8 12 18 24 36 48 72
75
50
Zymosan time course 2
0 2 4 8 12 18 24 36 48 72
75
50
Serum transferrin (full-length)
B
0 2 4 8 12 18 24 36 48 72
75
50
0 2 4 8 12 18 24 36 48 72
75
50
Peritoneal transferrin (full-length)
C
0 2 4 8 12 18 24 36 48 72
37
25
0 2 4 8 12 18 24 36 48 72
37
25
Peritoneal transferrin (cleaved)
D
0 2 4 8 12 18 24 36 48 72
50
37
0 2 4 8 12 18 24 36 48 72
50
37
Ovalbumin
Zymosan time course 3
0 2 4 8 12 18 24 36 48 72
75
50
Zymosan time course 4
0 2 4 8 12 18 24 36 48 72
75
50
Serum transferrin (full-length)
B
0 2 4 8 12 18 24 36 48 72
75
50
0 2 4 8 12 18 24 36 48 72
75
50
Peritoneal transferrin (full-length)
C
0 2 4 8 12 18 24 36 48 72
37
25
0 2 4 8 12 18 24 36 48 72
37
25
Peritoneal transferrin (cleaved)
D
0 2 4 8 12 18 24 36 48 72
50
37
0 2 4 8 12 18 24 36 48 72
50
37
Ovalbumin

**Figure 3.3 – Zymosan induces endogenous cleavage of full-length transferrin *in vivo*.** Goldfish were injected with 2.5 mg of zymosan (A) Serum samples were collected from the tail vein of individual goldfish at the indicated time point 0 – 72 hours post injection and probed for full-length transferrin, approximate molecular weight 65 – 67 kDa, using polyclonal rabbit antibody 9AG7. (B - C) Goldfish were subject to peritoneal lavage and supernatants collected at the indicated time point 0 – 72 hours post injection, corresponding to goldfish used in (A). Supernatants were probed for full-length (B) and cleaved-transferrin (C), approximate molecular weights 65 – 67 kDa and 33 – 37 kDa respectively, using 9AG7. (D) Peritoneal lavage Western blots were stripped and probed for ovalbumin using mouse monoclonal antibody (Santa Cruz Biotechnology), approximate molecular weight 42 kDa. Ovalabumin was added to 1X $PBS^{-/-}$ at a concentration of 10 $mg \bullet L^{-1}$ prior to peritoneal lavage as an introduced loading control.

A


Leucocytes/Lavage (x10^6)
20
15
10
5
0
0 2 4 8 12 18 24 36 48 72
Time (h)

B


% DHR+
100
75
50
25
0
0 2 4 8 12 18 24 36 48 72
Time (h)

C

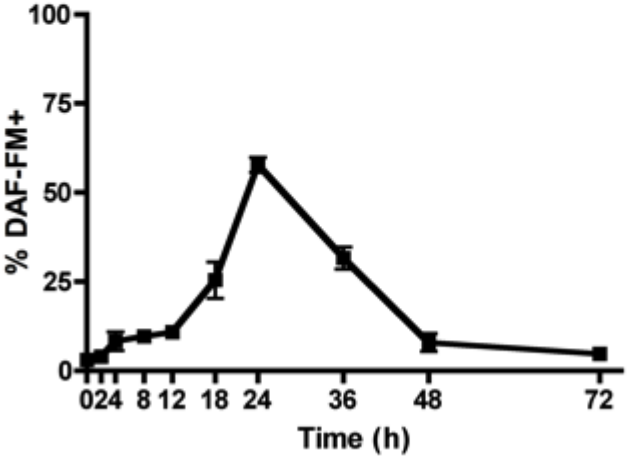

% DAF-FM+
100
75
50
25
0
0 2 4 8 12 18 24 36 48 72
Time (h)

**Figure 3.4 – Heat-killed *A. veronii* induces anti-microbial responses in goldfish leucocytes.** Goldfish were injected intraperitoneally with 5.0 x $10^6$ C.F.U. of heat-killed *A. veronii*. (A) Non-adherent leucocytes were isolated by peritoneal lavage and enumerated using a haemocytometer. (B) Respiratory burst in isolated leucocytes was determined using DHR. (C) NO in isolated leucocytes was determined using DAF-FM diacetate. n = 4

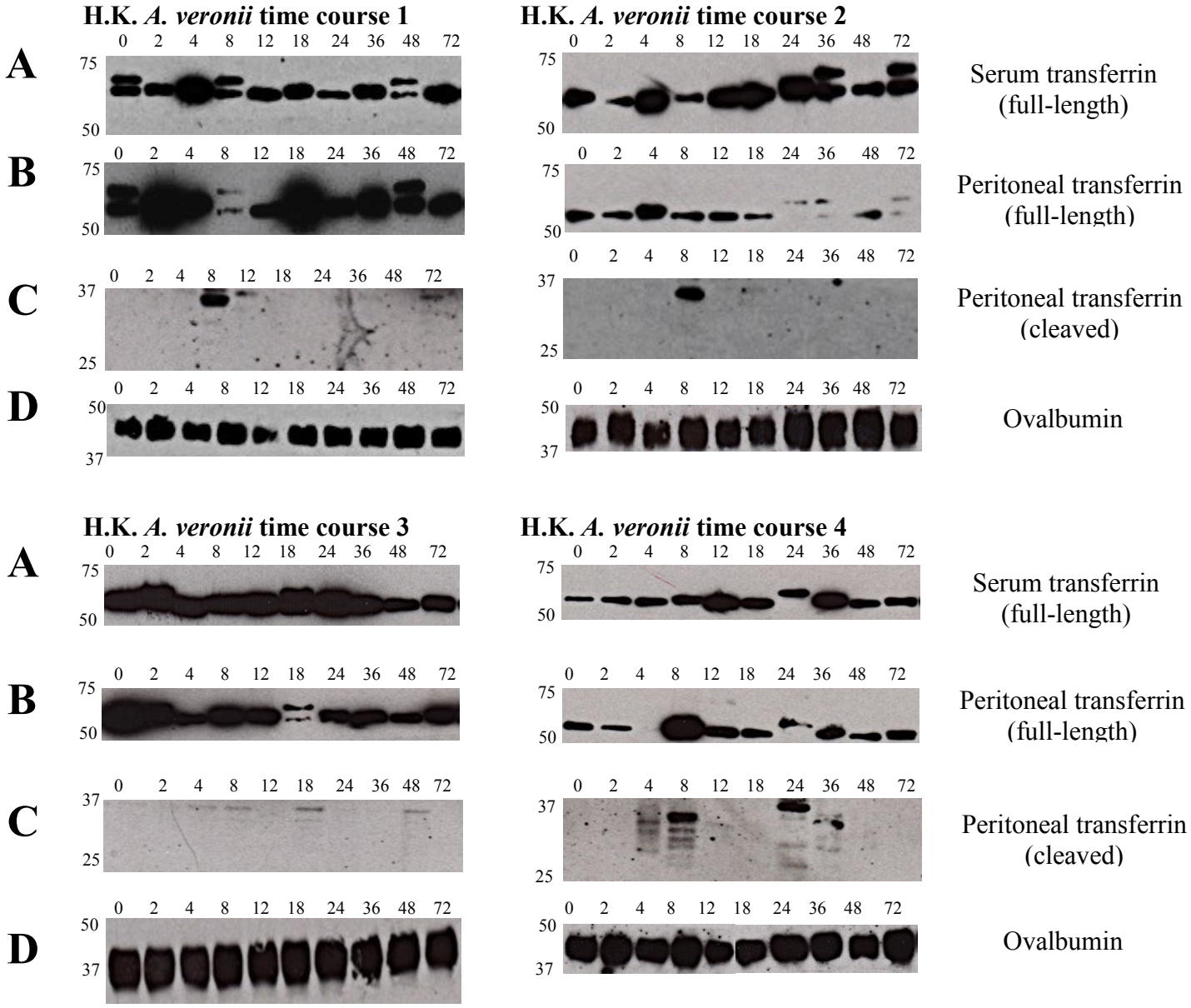
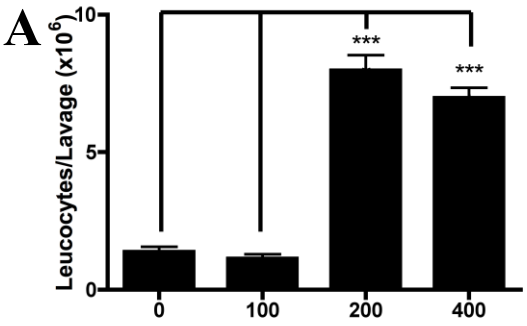
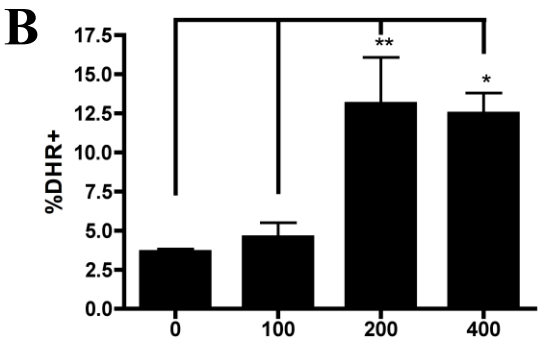
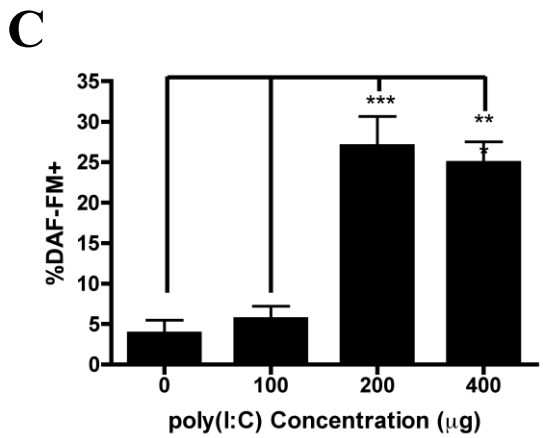

H.K. A. veronii time course 1
H.K. A. veronii time course 2
H.K. A. veronii time course 3
H.K. A. veronii time course 4
A
B
C
D
0 2 4 8 12 18 24 36 48 72
75
50
37
25
Serum transferrin (full-length)
Peritoneal transferrin (full-length)
Peritoneal transferrin (cleaved)
Ovalbumin

**Figure 3.5 – Heat-killed *A. veronii* induces endogenous cleavage of full-length transferrin *in vivo*.** Goldfish were injected with 5.0 x $10^6$ C.F.U. heat-killed *A. veronii*. (A) Serum samples were collected from the tail vein of individual goldfish at the indicated time point 0 – 72 hours post injection and probed for full-length transferrin, approximate molecular weight 65 – 67 kDa, using polyclonal rabbit antibody 9AG7. (B - C) Goldfish were subject to peritoneal lavage and supernatants collected at the indicated time point 0 – 72 hours post injection, corresponding to goldfish used in (A). Supernatants were probed for full-length (B) and cleaved-transferrin (C), approximate molecular weights 65 – 67 kDa and 33 – 37 kDa respectively, using 9AG7. (D) Peritoneal lavage Western blots were stripped and probed for ovalbumin using mouse monoclonal antibody (Santa Cruz Biotechnology), approximate molecular weight 42 kDa. Ovalbumin was added to 1X $PBS^{-/-}$ at a concentration of 10 mg•$L^{-1}$ prior to peritoneal lavage as an introduced loading control.


A
Leucocytes/Lavage (x10^6)
10
5
0
***
***
0
100
200
400


B
%DHR+
17.5
15.0
12.5
10.0
7.5
5.0
2.5
0.0
**
*
0
100
200
400


C
%DAF-FM+
35
30
25
20
15
10
5
0
***
**
*
0
100
200
400
poly(I:C) Concentration (μg)

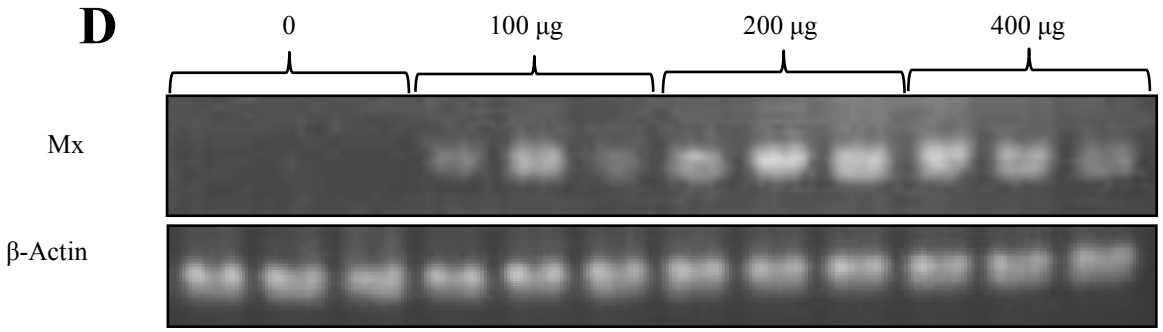
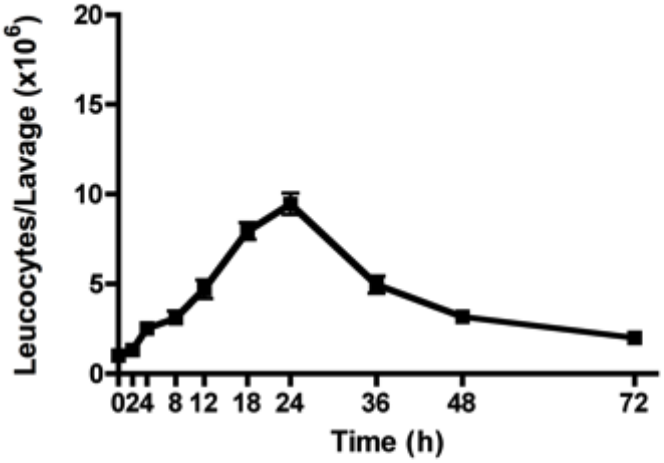
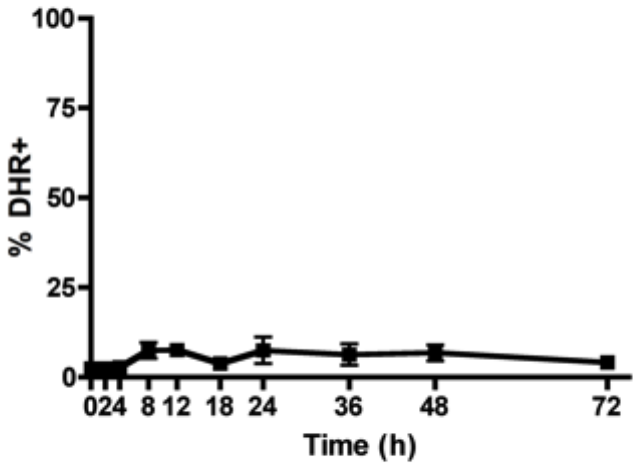

D
0
100 μg
200 μg
400 μg
Mx
β-Actin

**Figure 3.6 – 200 µg and 400 µg of poly(I:C) induces cellular infiltration, NO production and Mx gene expression in goldfish leucocytes**. Goldfish, *C. auratus*, were injected intraperitoneally with the indicted amount of poly(I:C). Non-adherent cells were harvested by peritoneal lavage 24 hours post injection. (A) Total leucocytes were enumerated using a haemocytometer. (B) Respiratory burst in isolated leucocytes was determined with DHR (bottom). (C) NO in isolated leucocytes was determined with DAF-FM diacetate. n = 4 (D) Mx gene expression was determined using Mx specific primers at RT-PCR 24 h post Poly(I:C) injection. n = 3. *$p<0.05$, **$p<0.01$, ***$p<0.005$ using 1-way analysis of variance.

**A**


Leucocytes/Lavage (x10^6)
20
15
10
5
0
0 2 4 8 12 18 24 36 48 72
Time (h)

**B**


% DHR+
100
75
50
25
0
0 2 4 8 12 18 24 36 48 72
Time (h)

**C**

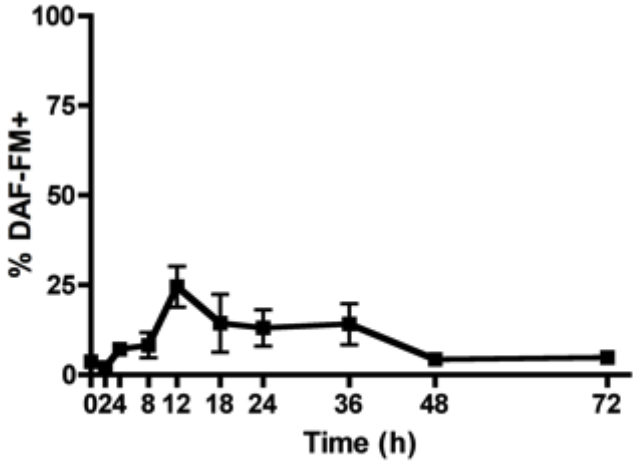

% DAF-FM+
100
75
50
25
0
0 2 4 8 12 18 24 36 48 72
Time (h)

**Figure 3.7 – poly(I:C) induces cellular infiltration and NO production in goldfish leucocytes.** Goldfish were injected intraperitoneally with 200 μg of poly(I:C) (A) Non-adherent leucocytes were isolated by peritoneal lavage and enumerated using a haemocytometer. (B) Respiratory burst in isolated leucocytes was determined using DHR. (C) NO in isolated leucocytes was determined using DAF-FM diacetate. n = 4

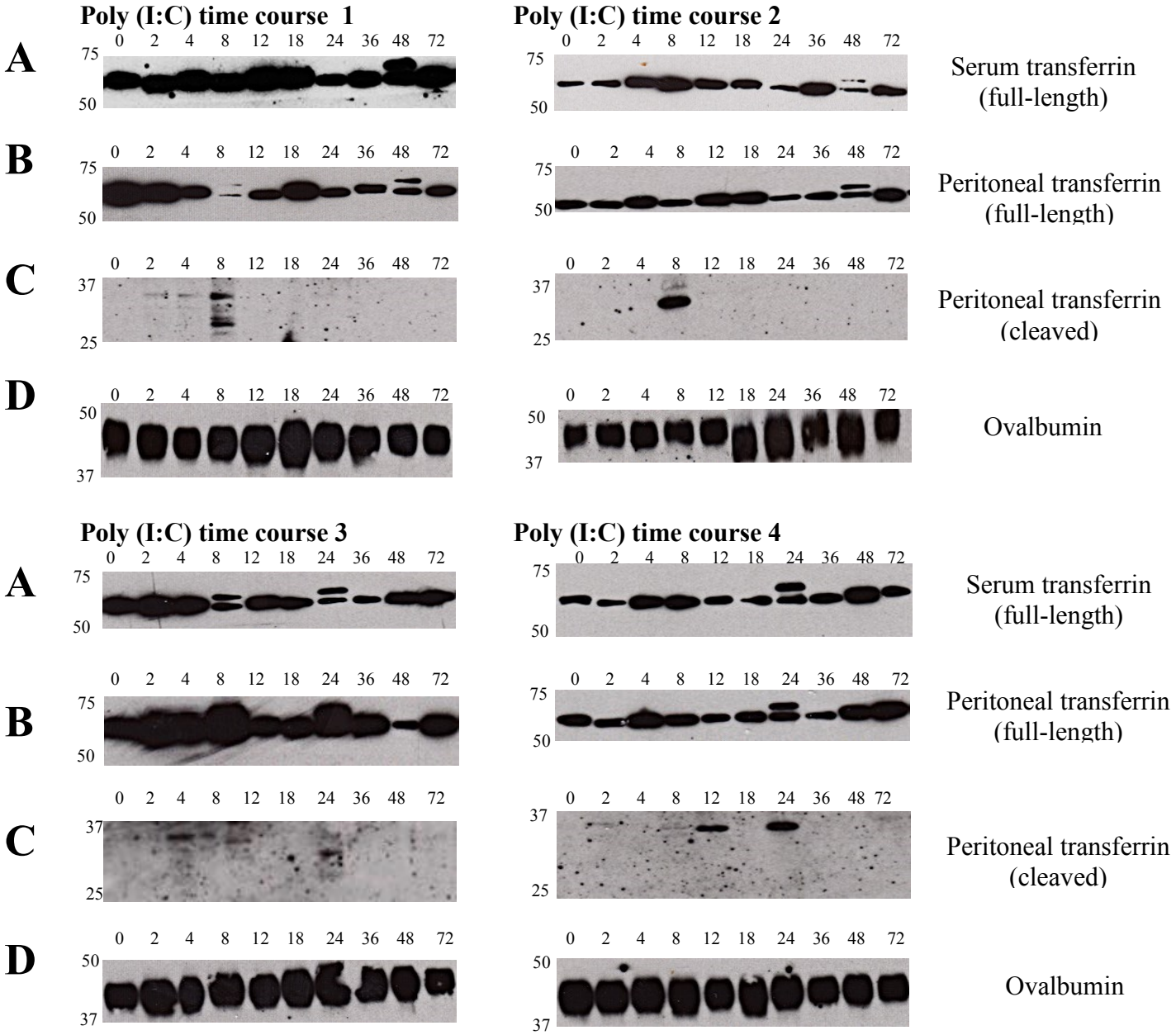

Poly (I:C) time course 1
Poly (I:C) time course 2
Poly (I:C) time course 3
Poly (I:C) time course 4
A
B
C
D
0 2 4 8 12 18 24 36 48 72
75
50
37
25
Serum transferrin (full-length)
Peritoneal transferrin (full-length)
Peritoneal transferrin (cleaved)
Ovalbumin

**Figure 3.8 – poly(I:C) induces endogenous cleavage of full-length transferrin *in vivo*.** Goldfish were injected with 200 μg of poly(I:C) (A) Serum samples were collected from the tail vein of individual goldfish at the indicated time point 0 – 72 hour post injection and probed for full-length transferrin, approximate molecular weight 65 – 67 kDa, using polyclonal rabbit antibody 9AG7. (B - C) Goldfish were subject to peritoneal lavage and supernatants collected at the indicated time point 0 – 72 hours post injection, corresponding to goldfish used in (A). Supernatants were probed for full-length (B) and cleaved-transferrin (C), approximate molecular weights 65 – 67 kDa and 33 – 37 kDa respectively, using 9AG7. (D) Peritoneal lavage Western blots were stripped and probed for ovalbumin using mouse monoclonal antibody (Santa Cruz Biotechnology), approximate molecular weight 42 kDa. Ovalabumin was added to 1X $PBS^{-/-}$ at a concentration of 10 $mg \bullet L^{-1}$ prior to peritoneal lavage as an introduced loading control

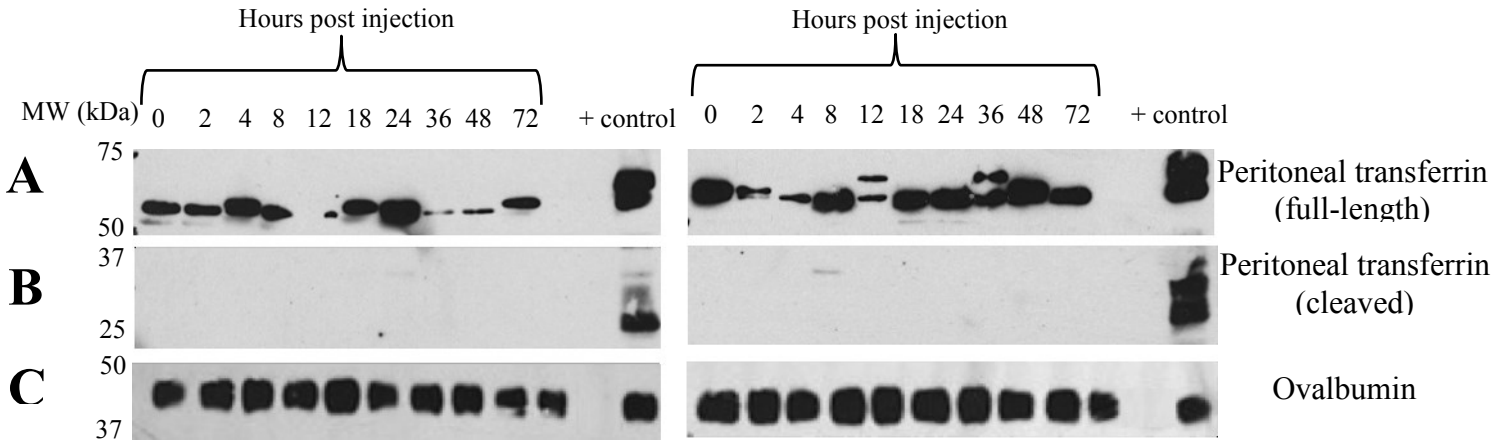



**Figure 3.9 – Injection of 1X PBS$^{-/-}$ does not produce transferrin cleavage products**. *C. auratus* were injected with 100 µl of 1X PBS$^{-/-}$. Goldfish were subject to peritoneal lavage and supernatants collected at the indicated time point 0 – 72 hours post injection. Supernatants were probed for (A) full-length and (B) cleaved-transferrin, approximate molecular weights 65 – 67 kDa and 33 – 37 kDa respectively, using 9AG7. (C) Peritoneal lavage Western blots were stripped and probed for ovalbumin using mouse monoclonal antibody (Santa Cruz Biotechnology), approximate molecular weight 42 kDa. Ovalabumin was added to 1X PBS$^{-/-}$ at a concentration of 10 mg•L$^{-1}$ prior to peritoneal lavage as an introduced loading control.

# Chapter 4: Production of transferrin cleavage products during acute inflammation in goldfish, *C. auratus*

## 4.1 Introduction

Transferrin is a bi-lobed monomeric glycoprotein, of approximately 70 – 80 kDa, that is well known for its role in iron transport (Dautry-Varsat et al., 1983; Gkouvatsos et al., 2012; Harding et al., 1983; Johnson and Wessling-Resnick, 2012). Transferrin is part of a larger family of iron transport proteins that includes lactoferrin (Farnaud and Evans, 2003) ovotransferrin (Giansanti et al., 2012), and melanotransferrin (Dunn et al., 2006). This molecule is believed to have evolved from a gene duplication event over 670 million years ago, due to the high homology of amino acid sequence, and tertiary structure between the N- and C-lobes (Lambert et al., 2005). The structure of transferrin is highly conserved throughout evolution with transferrins from different species sharing high structural and sequence homology (greater than 70% within mammalian lineages and 25 – 30% primary amino acid sequence identity with insect transferrin) (Baker et al., 2002; Huebers et al., 1988). More recently, there has been a growing appreciation for the role of transferrin in host defence. These include sequestration of iron from invading pathogens (Ellis, 2001; Skaar, 2010), direct killing of bacterial pathogens (Ibrahim et al., 1998), contributions as an acute phase protein primarily inhibiting growth and proliferation of pathogens (Bayne and Gerwick, 2001; Kovacevic et al., 2015), and activation of anti-microbial responses in macrophages *in vitro* (Jurecka et al., 2009; Stafford and Belosevic, 2003; Stafford et al., 2001). Notably, these contributions do not appear to be limited to transferrin, as other members of this family of proteins are also able to modulate inflammatory responses. For example, lactoferrin is synthesized and

released by cells undergoing apoptosis resulting in decreased neutrophil chemotaxis (Bournazou et al., 2009; Poon et al., 2014). Ovotransferrin has also been found to modulate IL-6, ROI and RNI production in avian macrophages as well as heterophil degranulation *in vitro* (Xie et al., 2002).

Serum transferrin is primarily synthesized in the liver and secreted into the blood (Lieu et al., 2001). Recently, it has been shown that, upon activation with inflammatory stimuli, teleost macrophages also express transferrin *in vitro* (Stafford and Belosevic, 2003). In mammals full-length transferrin is approximately 80 kDa while in teleost fish the native transferrin is a polymorphic protein of 60 – 70 kDa (Yang et al., 2004). Upon cleavage teleost transferrin yields immunologically active peptides of 30 – 40 kDa (Stafford and Belosevic, 2003; Stafford et al., 2001). These cleavage products are conserved, with similarly sized transferrin breakdown products generated by *Pseudomonas aeruginosa* elastase in the bronchoalveolar lavage fluid of human cystic fibrosis patients, likely contributing to pathology (Britigan et al., 1993). Indeed, it has been shown that bovine and teleost cleavage products can induce anti-microbial responses in bovine and teleost macrophage systems, and vice-versa (Haddad and Belosevic, 2009; Stafford and Belosevic, 2003). The primary enzymes responsible for facilitating transferrin cleavage has not yet been identified, however elastase, trypsin, and chymotrypsin have all been shown to yield transferrin break down products (Haddad and Belosevic, 2009; Stafford, 2003). The resultant peptide fragments from different polymorphic variants of native teleost transferrin yield varying degrees of NO production (Jurecka et al., 2009). However, the ability of transferrin cleavage products to induce

anti-microbial responses appears to be the result of a conserved peptide sequence in the N-lobe that can cross react between species (Haddad and Belosevic, 2009).

In this section of my thesis the goal for this study was to assess the production of transferrin cleavage products during the acute inflammatory process *in vivo*. It was hypothesized that cleaved transferrin products would be produced locally within an immune challenge site, contribute to antimicrobial responses of infiltrating leucocytes and display differential kinetics depending on the inflammatory stimulus. To test this hypothesis, I examined the role of transferrin *in vivo* using a self-resolving peritonitis model in goldfish, *Carassius auratus,* where acute inflammation was triggered via sterile or pathogen driven challenges. Both local and systemic immune responses were considered. A combinatorial gene expression- and protein analysis-based approach demonstrated a role for cleaved transferrin during teleost acute inflammation. In addition to transferrin being expressed at the inflammatory site by macrophages, I show endogenous transferrin cleavage products at the inflammatory site, which are not seen in the serum. Finally, I show that these resultant peptides are selectively produced during pathogen induced, but not sterile, inflammation.

## 4.2 Results

### 4.2.1 Transferrin is expressed by goldfish macrophages *in vivo*

It has been shown previously that mitogen activated total kidney leucocytes do not express transferrin, however cultured primary kidney macrophages express transferrin as early as 1 hour post stimulation *in vitro* (Stafford and Belosevic, 2003). The expression of transferrin by macrophages has also been shown in insects, birds, and mammals (Djeha et al., 1995; Xie et al., 2002; Yoshiga et al., 1997). To investigate the leucocyte

contributions to local production of transferrin during an inflammatory process, I first used RT-PCR and transferrin specific primers to detect the time point of maximum transferrin gene expression by infiltrating leucocytes following *in vivo* peritoneal stimulation with zymosan as a pathogen mimic (Figure 4.1). Kinetic analysis indicated that the macrophage subset upregulated transferrin expression as early as 8 hours following zymosan intraperitoneal injection, with an additional increase by 24 hours (Figure 4.1A). Expression of transferrin by peritoneal macrophages subsequently decreased by 48 hours post challenge, which corresponds to the resolution phase of acute inflammation in this animal challenge model (Havixbeck et al., 2016). In further evaluating the inducible expression of transferrin by the macrophage enriched population it was evident that transferrin expression is increased with pathogen-challenge, but not sterile challenge (Figure 4.1B). Additionally, I found that macrophages, but not neutrophils, expressed transferrin following challenge, at a period corresponding to the peak of the acute inflammatory process (18 hours; Figure 4.1C; Havixbeck et al., 2016). A HEMA3 cytochemical stain of isolated cell populations confirmed the presence of leucocytes with classical macrophage and neutrophil morphology, respectively (Figure 4.1D). This was further supported by classical flow cytometric profiles for macrophage and neutrophil enriched isolates, respectively (Figure 4.1E).

### 4.2.2 Transferrin is cleaved by a endogenous enzyme at the inflammatory site

The transferrin-cleaving ability of endogenous leucocyte derived enzymes has been previously described (Jurecka et al., 2009; Stafford and Belosevic, 2003; Stafford et al., 2001). To further characterize the enzymes responsible for transferrin cleavage I isolated various protein and leucocyte preparations from the inflammatory in vivo

challenge site and examined their ability to cleave full-length transferrin. Both non-adherent leucocytes and soluble mediators from the site of inflammation were collected. I used an *ex vivo* approach, incubating bovine transferrin with various cell-free preparations to demonstrate the transferrin cleavage ability of a secreted enzymes (Figure 4.2A). This active enzyme is present only at the inflammatory site, and not present in the serum. Transferrin cleavage products were detected as early as 8 h, and present as late as 24 h post-stimulation with zymosan (Figure 4.2B). By 48 hours post-stimulation I was not able to detect transferrin cleavage products.

I then incubated bovine transferrin with lysed macrophage and neutrophil preparations to examine the potential for a leukocyte source of transferrin cleaving capacity at the inflammatory site. I found that multiple cell types can contribute to transferrin cleavage at the inflammatory site (Figure 4.2C). This is consistent with previous observations that show cell lysates from naïve cell populations (i.e. primary kidney leucocytes) and *in vitro* derived primary kidney macrophage cultures can facilitate transferrin cleavage (Jurecka et al., 2009; Stafford and Belosevic, 2003). Interestingly, the ability to cleave transferrin using cellular lysates was seen only when live cells isolated from the inflammatory site were used. In contrast, lysates derived from primary kidney leucocyte apoptotic cell preparations did not yield detectable transferrin cleavage products (Figure 4.2C).

To confirm that enzymes were responsible for facilitating transferrin cleavage three preparations, and a negative control were analysed where the preparations were subject to enzyme inaction and inhibition. It was evident that porcine elastase, inflammatory leucocytes, and inflammatory supernatants posses the ability to cleavage

transferrin, albeit in different amounts (Figure 4.2D). However when these preparations were denatured (boiled) or inhibited (protease inhibitors) they were unable to facilitate detectable levels of transferrin cleavage.

### 4.2.3 Goldfish peritoneal leucocytes internalize or degrade bovine transferrin cleavage products *ex vivo*

To examine the potential for leucocytes to process transferrin cleavage products I incubated inflammatory leucocytes with bovine transferrin cleavage products as generated earlier (4.2.2). These leucocytes were incubated with the bovine transferrin cleavage preparation for the indicated time point and then the supernatants were analysed by Western blotting to examine the disappearance of transferrin cleavage products (Figure 4.3). The amount transferrin cleavage products begin to decrease as early as 30 minutes and are not detected by 1 hour post incubation. Controls omitting leucocytes indicated that the transferrin cleavage products do undergo significant degradation during the additional 2 hours incubation period.

### 4.2.4 Transferrin products are present at the inflammatory site *in vivo*

To evaluate the presence of transferrin cleavage products at the inflammatory site I evaluated peritoneal exudate collected 18 hours post zymosan injection, corresponding to the peak pro-inflammatory response in goldfish (Havixbeck et al., 2016). I show for the first time endogenously produced transferrin cleavage products *in vivo* present only at an inflammatory site (Figure 4.4) but not in the serum (data not shown). I also note that cleaved transferrin products were present only in pathogen-induced, but not sterile inflammation. Both native forms of transferrin were seen in serum and the peritoneum (Figure 4.4). I was able to detect polymorphic variants in the native form observed

previously (Yang et al., 2004). The pattern of cleavage products collected *in vivo* also display a large degree of heterogeneity compared to pattern observed in other teleost systems (Stafford et al., 2001) (Figure 4.4).

### 4.2.4 Transferrin cleavage are produced in pathogen-induced, but not sterile inflammation

The addition of exogenous transferrin has been shown to strengthen the anti-microbial response of cultured goldfish macrophages to a diverse array of different pathogens, and pathogen products (Stafford and Belosevic, 2003). To evaluate the presence of endogenously produced cleaved transferrin products under multiple inflammatory conditions I compared sterile and pathogen-induced processes and further used a suite of non-proliferative pathogen-derived inflammatory triggers (Figures 4.4 and 4.5). I determined that cleaved transferrin products were produced only in pathogen induced, but not sterile inflammation (Figure 4.4). Further, transferrin cleavage products were differentially generated based on the non-proliferative pathogen-derived inflammatory trigger used. Whereas zymosan and heat-killed *A. veronii* intraperitoneal challenge yielded robust production of transferrin cleavage products at the earliest time point examined post-challenge (8 hours), poly(I:C) only showed detectable production of transferrin cleavage products 24 hours post-challenge (Figure 4.6A-C).

Given that transferrin cleavage products have been reported to play a role in the induction of leucocyte anti-microbial responses (Jurecka et al., 2009; Stafford and Belosevic, 2003; Stafford et al., 2001) I investigated the kinetics of these leucocyte mediated responses, attempting to correlate them with the appearance of transferrin cleavage products. To evaluate the leucocyte mediated responses to multiple pathogenic stimuli I used zymosan in addition to heat-inactivated bacteria - *Aeromonas veronii*, and

the viral mimic Poly(I:C) (Figure 4.6) to induce inflammation and then evaluate the kinetics of anti-microbial responses. I found that the patterns of leucocyte infiltration to the inflammatory site among the three pathogen-challenges exhibited marked differences with leucocyte infiltration reaching maximum at 18 hours for zymosan and *A. veronii* but 24 hours, for Poly(I:C) (Figure 4.6A). I also noticed that the pattern of anti-microbial responses (i.e. respiratory burst and NO) between zymosan, *A. veronii*, and Poly(I:C) were significantly different with robust respiratory burst (Figure 4.6B) and NO (Figure 4.6C) responses when using zymosan and *A. veronii*, whereas Poly(I:C) only induced minimal NO responses and did not appear to induce a respiratory burst response.

## 4.3 Discussion

The presence of endogenously derived transferrin cleavage products *in vivo* confirms previous observations, from *in vitro* cell culture systems, that cleaved transferrin fragments may contribute to acute inflammation by activating macrophages (Haddad and Belosevic, 2009; Jurecka et al., 2009; Stafford and Belosevic, 2003; Stafford et al., 2001). An endogenous soluble enzyme is produced at the acute inflammatory site that contributes a diverse array of transferrin cleavage products detectable by Western blot. These are preferentially produced following pathogen-driven inflammation and not sterile inflammation. Further, differential kinetics for production of transferrin cleavage products are exhibited following different pathogen derived triggers. It remains to be determined if these differential kinetics reflect specialization of the transferrin cleavage response to different pathogens. Alternatively, it may reflect differential sensitivity of the host to each of the *in vivo* triggers used (Poynter and DeWitte-Orr, 2015).

Previous work shows the ability of transferrin cleavage products to induce anti-microbial responses are associated with conserved peptide sequence in the N-lobe that can cross react between species (Haddad and Belosevic, 2009). However, based on the significant heterogeneity observed in the pattern of transferrin cleavage products observed at the peritoneal challenge site, it remains unclear if a multitude of non-functional fragments are generated to liberate this key conserved functional sequence, or whether multiple products with perhaps distinct antimicrobial functions are generated during the transferring cleavage process *in vivo*. The generation of several transferrin cleavage products detected by Western blot could be due to a multitude of factors. One contributing factor is that there are a diverse array of enzymes secreted at the inflammatory site both by inflammatory leucocytes and the adherent cell populations that could facilitate the generation of multiple breakdown products (Gerwitz et al., 2001). In addition there are three known isoforms of the full-length transferrin in *C. auratus* (Yang et al., 2004) that could result in breakdown products of various molecular weights. It is noteworthy that the cleavage products detected by using the rabbit anti-carp transferrin polyclonal primary antibody are representative of several transferrin fragments generated, of which smaller fragments are not detected by Western blot but have been previously characterized by mass spectrometry (Haddad and Belosevic, 2009). Despite the fact that the immunologically active transferrin-derived peptide sequence, TMAP, has previously been identified (Haddad and Belosevic, 2009) it is possible that additional functional transferrin breakdown products are generated, contributing to leucocyte responses through engagement of multiple receptors. These functional fragments will need to be

further defined as additional reagents (e.g. antibodies to specific cleavage products) become available to increase our resolution capacity to this biological phenomenon.

Our results add to previous observations that activated macrophages increase expression of transferrin in response to inflammatory stimuli (Stafford and Belosevic, 2003). This increase in expression was also seen only in pathogen-driven, but not sterile inflammation, with transferrin gene expression by macrophage enriched populations increasing in response to pathogen-challenge. However, we show that teleost peritoneal macrophages constitutively express a basal level of transferrin prior to activation with inflammatory stimuli. Previous work showed that non-activated cultured teleost macrophages did not express transferrin, but expressed transferrin as early as 1 hour post stimulation (Stafford and Belosevic, 2003). However, *in vivo*, murine peritoneal macrophages display a low level of basal transferrin expression that is up-regulated with cytokine stimulation (Djeha et al., 1995). It was evident that inflammatory neutrophils did not express full-length transferrin, however this does not preclude neutrophils from expressing an alternate splice variant of a truncated from of transferrin.

Previous studies have shown that an unknown leucocyte derived enzyme, present in cell lysate preparations and supernatants of mitogen stimulated kidney leucocytes can cleave transferrin (Stafford and Belosevic, 2003; Stafford et al., 2001). Our recent work has shown that in a model of zymosan induced peritonitis goldfish neutrophils are the primary infiltrating leucocyte, comprising approximately 50% of the leucocyte population at the peak pro-inflammatory phase (Havixbeck et al., 2016). These teleost neutrophils also possess distinct immunoregulatory phenotypes, with early-infiltrating neutrophils displaying a distinct pro-inflammatory phenotype, whereas neutrophils at

later stages of the response display a pro-resolution phenotype (Havixbeck et al., 2016). However it was evident that there was no significant difference in the capacity of pro-inflammatory and pro-resolving neutrophil lysates to generate transferrin cleavage products. Indeed, this ability was also present with early- and late-infiltrating macrophage and monocyte populations, suggesting that these leucocytes subsets posses enzymes capable of facilitating transferrin cleavage. However, given the significantly greater number of neutrophils present during the peak pro-inflammatory response we propose that neutrophils are the primary cell type responsible for transferrin cleavage *in vivo*. Although the identity of the enzyme(s) capable of producing transferrin cleavage products remains elusive one enzyme that is secreted in copious amounts at the inflammatory site by both pro-inflammatory neutrophils, and macrophages is elastase (Faurschou and Borregaard, 2003; Takemura and Werb, 1984). Previous work has also shown that elastase can cleave goldfish transferrin into immunostimulatory fragments (Stafford, 2003). Elastase is a well-established component of secretory neutrophil granules (Faurschou and Borregaard, 2003) and is a significant component of neutrophil extracellular traps, NETs (Yipp et al., 2012).

Zymosan induced peritonitis in goldfish has been shown to serve as a robust model to examine both the pro-inflammatory and resolution phases of the acute inflammatory response. However we look to characterize the ability of multiple pathogenic triggers to generate transferrin breakdown products, and correlate the appearance of these products with the microenvironment at the inflammatory site with three classical markers of acute inflammation (leucocyte influx, respiratory burst, and NO). Similar to recent work we found that zymosan induced robust leucocyte influx and

respiratory burst responses (Havixbeck et al., 2016; Rieger et al., 2015). Additionally we characterized the nitric oxide response of infiltrating leucocytes, finding a delay in the peak NO response compared to leucocyte influx and respiratory burst, shifting from 18 hours post injection to 24 hours post injection. This delay is likely the result of leucocytes having to synthesize inducible nitric oxide synthase (iNOS) *de novo* to produce significant quantities of anti-microbial NO (Korhonen et al., 2005). The differential capacity of the different pathogens and pathogen-mimics to induce varying levels of antimicrobial responses could reflect the differential sensitivity of the host to the suite of inflammatory-challenges used in these experiments (Poynter and DeWitte-Orr, 2015). Interestingly, we note that transferrin cleavage products were only detected at the latest time point examined using poly(I:C), however there were minimal respiratory burst and nitric oxide responses. This presents this presents the possibility that induction of respiratory burst and nitric oxide are not pre-requisites for the generation of transferrin cleavage products (for example both respiratory burst and NO production are low at 24 hours post-injection of Poly(I:C) yet we still see generation of transferrin cleavage products (Figure 4.5, Figure 4.6B, C) . However in all cases the presence of transferrin cleavage products correlates with leucocyte infiltration (Figure 4.5, Figure 4.6A).

Transferrin has previously been shown to act as an acute phase protein produced in response to bacterial infection. This mechanism is highly conserved being observed in diverse systems, including arthropods (Yoshiga et al., 1999; Yoshiga et al., 1997), teleost fish (Bayne and Gerwick, 2001), and humans (Cray et al., 2009). Although in fish and mammals transferrin is primarily produced in the liver (Bayne and Gerwick, 2001; Lieu et al., 2001) increased production of transferrin by resident macrophages may represent a

primitive innate defence mechanism developed in lower evolutionary lineages (Yoshiga et al., 1999; Yoshiga et al., 1997). Indeed, it has been observed that pathogens have adapted various mechanisms to combat host-iron sequestration by degradation of host transferrin in diverse groups. *Pseudomonas aeruginosa* derived elastase has been shown to degrade transferrin making more iron available to proliferating pathogens in humans (Britigan et al., 1993), whereas *Trypanoplasma borreli* will internalize and digest transferrin in its lysosomal compartment, eventually releasing transferrin cleavage products back into the host, the common carp, *Cyprinus carpio* (Ruszczyk et al., 2008). The ability of transferrin cleavage products to activate macrophages, combined with its appearance with multiple pathogenic stimuli, adds to a growing body of evidence that suggests cleaved transferrin belong to a diverse suite of self-derived DAMP's, which include heat-shock proteins, fibronectin fragments, lactoferrin fragments, and hyaluronan fragments (Zhang and Mosser, 2008). On the other hand, it remains unclear whether activation of macrophages by endogenously cleaved transferrin is an adaptation acquired by teleost fish or an ancient conserved mechanism present in other taxa. It also remains unclear whether there is a differential role for macrophage-produced transferrin at the inflammatory site compared to serum transferrin. Macrophage-produced transferrin may act in an autocrine/paracrine fashion to induce the early macrophage-facilitated events during acute inflammation, which are then propagated with the influx of serum transferrin and inflammatory leucocytes upon activation.

This study indicates that cleaved transferrin products contribute to acute inflammation in teleost fish. Given the conservation of transferrin or transferrin-like molecules, as well as macrophages or macrophage-like cells across the animal kingdom,

cleaved transferrin products may represent conserved immunostimulatory molecules belonging to the “alarmins” family of molecules. This study provides insight into a potentially primitive mechanism of inflammatory control present in lower vertebrates in addition to the diverse roles of transferrin, and members of the transferrin family of proteins, in immunoregulation.

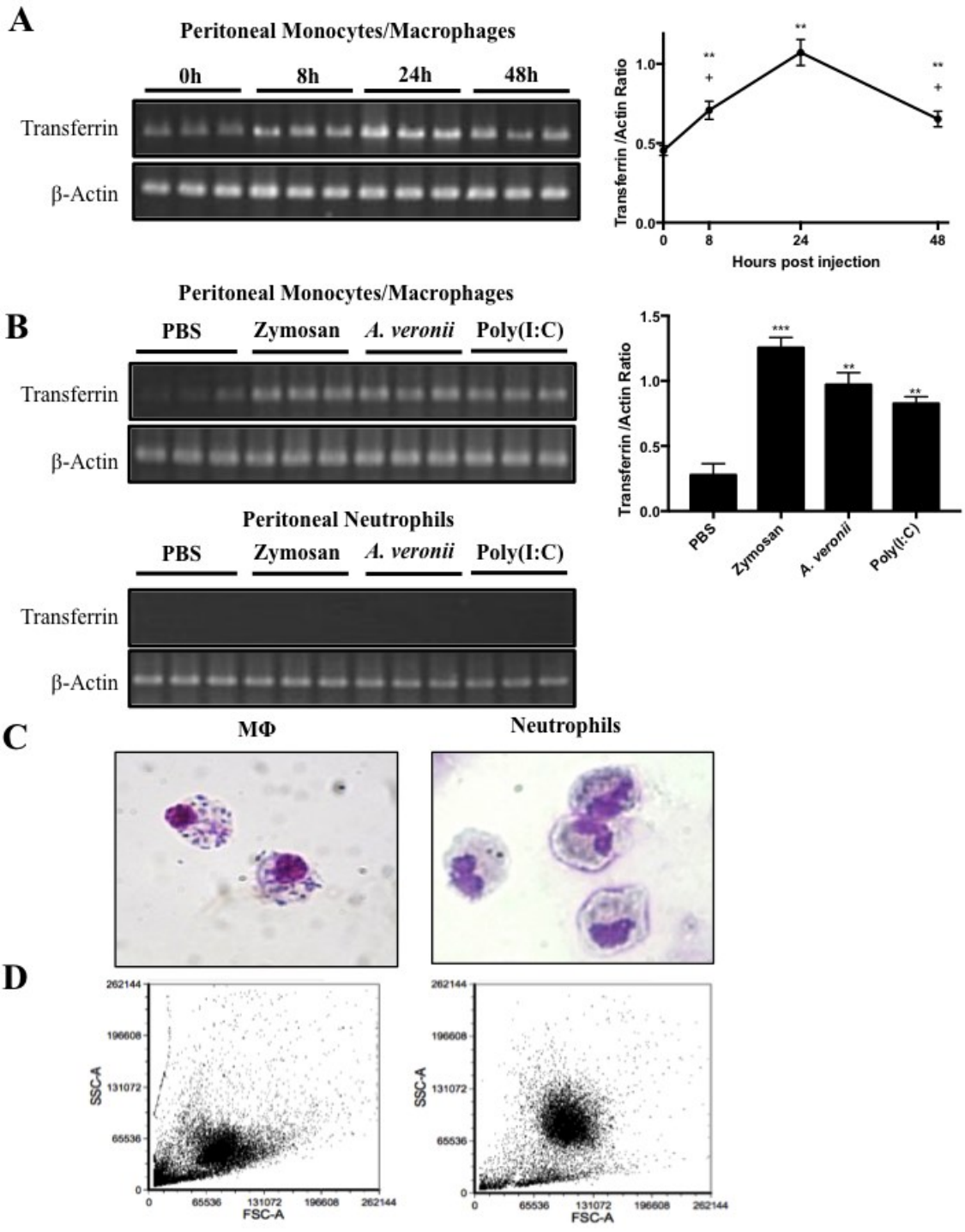

A
Peritoneal Monocytes/Macrophages
0h
8h
24h
48h
Transferrin
β-Actin
Transferrin /Actin Ratio
Hours post injection
B
Peritoneal Monocytes/Macrophages
PBS
Zymosan
A. veronii
Poly(I:C)
Transferrin
β-Actin
Peritoneal Neutrophils
Transferrin /Actin Ratio
C
MΦ
Neutrophils
D
SSC-A
FSC-A

**Figure 4.1 – Transferrin is expressed by macrophages, but not neutrophils, at the inflammatory site**. RT-PCR was used to detect the expression of transferrin mRNA in goldfish peritoneal leucocytes. (A) Macrophages were isolated at the indicated time point after injection with zymosan and PCR-amplified with transferrin specific primers. Densitometry was used to quantify differences in transferrin band intensity (B) Macrophages and neutrophils were isolated 24 hours after injection with the indicated stimulus and PCR-amplified with transferrin specific primers. Densitometry was used to quantify differences in transferrin band intensity. (C) Modified Wright-Giesma straining and (D) flow cytometry FACS plots were used to asses purity of isolated leucocyte populations. *$P<0.05$ using 1-way analysis of variance compared to non-injected, **$P<0.01$ using 1-way analysis of variance compared to non-injected, +$P<0.05$ using 1-way analysis of variance compared to 24 hours post-injection.

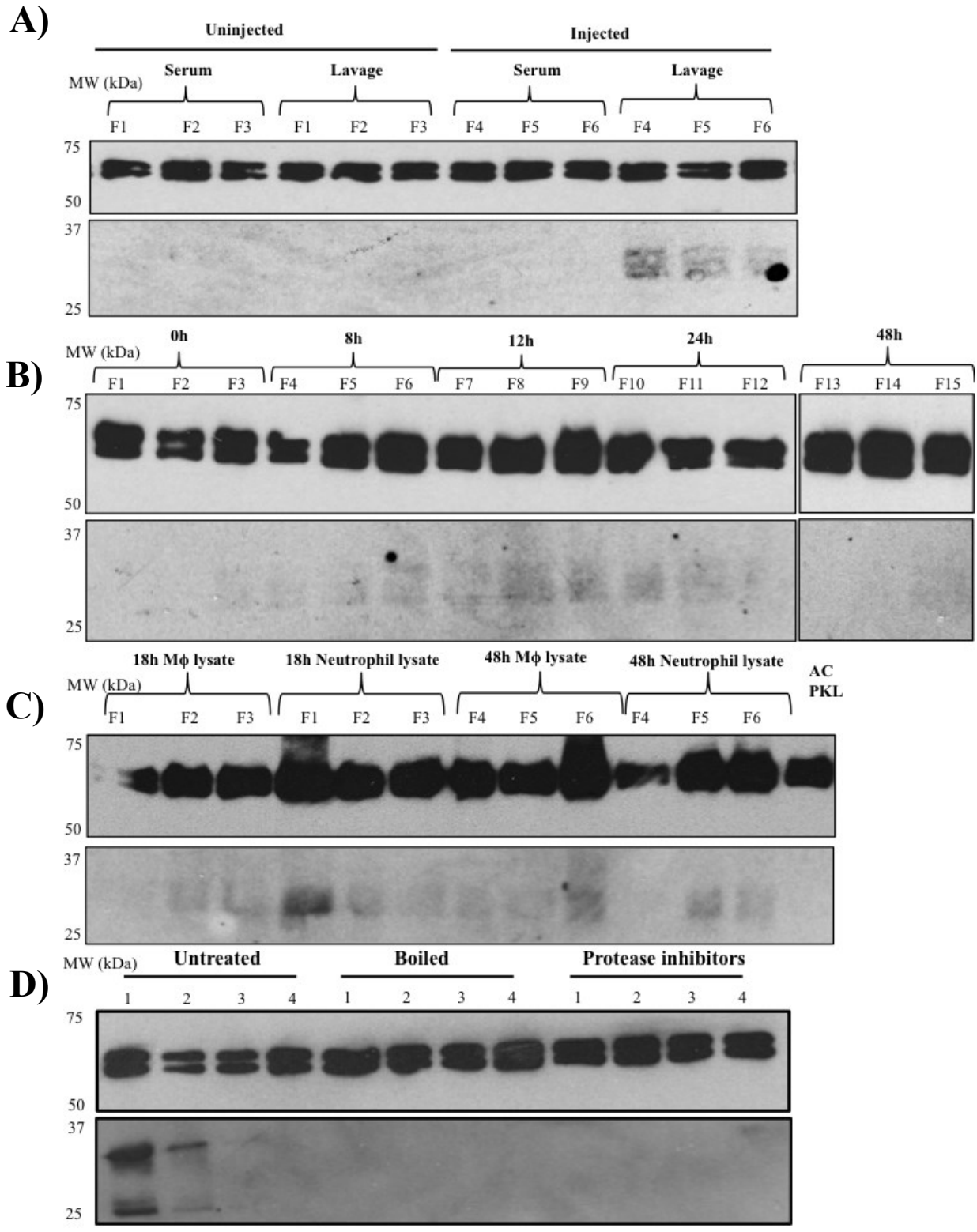

A)
Uninjected
Injected
MW (kDa)
Serum
Lavage
Serum
Lavage
F1 F2 F3 F1 F2 F3 F4 F5 F6 F4 F5 F6
75
50
37
25
B)
MW (kDa)
0h
8h
12h
24h
48h
F1 F2 F3 F4 F5 F6 F7 F8 F9 F10 F11 F12 F13 F14 F15
75
50
37
25
C)
18h Mϕ lysate
18h Neutrophil lysate
48h Mϕ lysate
48h Neutrophil lysate
AC
PKL
MW (kDa)
F1 F2 F3 F1 F2 F3 F4 F5 F6 F4 F5 F6
75
50
37
25
D)
MW (kDa)
Untreated
Boiled
Protease inhibitors
1 2 3 4 1 2 3 4 1 2 3 4
75
50
37
25

**Figure 4.2 – Goldfish leucocytes produce a soluble mediator capable of contributing to transferrin cleavage**. Goldfish were injected with 2.5 mg of zymosan and then various preparations were incubated with 2.5 $\mu g \bullet mL^{-1}$ of commercially purchased bovine holo-transferrin for 4 hours at 20 °C. Samples were separated by SDS-PAGE and analysed by Western blot using commercially purchased bovine-transferrin polyclonal antibody as described in section 2. (A) Serum samples and peritoneal lavage fluid was collected from goldfish injected with 2.5 mg of zymosan 18 h post injection and incubated with bovine transferrin. (B) Peritoneal lavage fluid supernatants were collected from zymosan-injection fish at the indicated time point and incubated with bovine transferrin. (C) Macrophages and neutrophils were isolated at the indicated time point post zymosan-injection, lysed, and incubated with bovine transferrin. AC PKL – Apoptotic primary kidney leucocytes. (D) Bovine holo-transferrin was incubated with various preparations to evaluate transferrin-cleaving ability. Preparations were: 1 – Porcine elastase, 2 – peritoneal leucocyte lysate, 3 – peritoneal supernatant, 4 – 1X $PBS^{-/-}$.

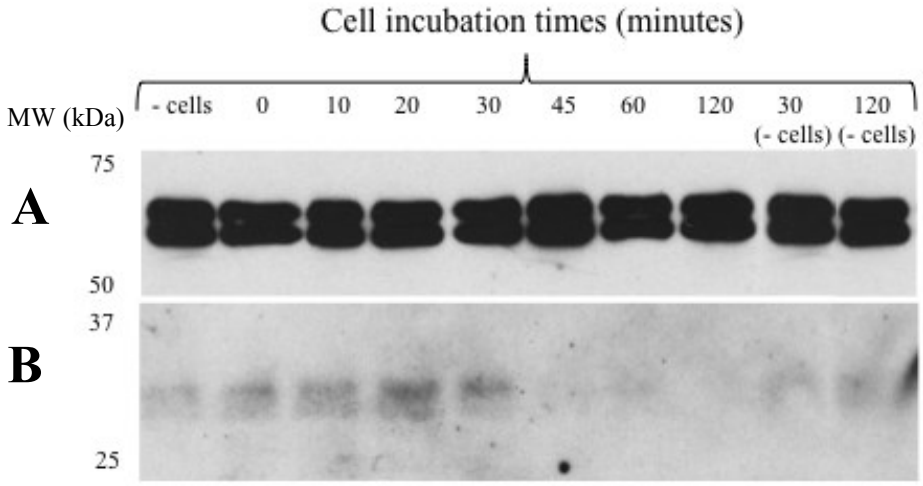



**Figure 4.3 – Goldfish leucocytes internalize or degrade transferrin cleavage products *ex vivo***. Goldfish were subject to peritoneal lavage with 3 mL of ice-cold 1X $PBS^{-/-}$ 18 hours post injection. Lavage fluid was centrifuged at 400g for 10 minutes and supernatants collected. Peritoneal lavage supernatant samples were incubated with 2.5 $\mu g \bullet mL^{-1}$ commercially purchased bovine holo-transferrin for 3h at 20 °C. A second goldfish was then subject to peritoneal lavage with 10 mL of ice-cold 1X $PBS^{-/-}$ 18 h post injection, and leucocytes enumerated using a haemocytometer. 1.0 x $10^6$ leucocytes were then added to each tube of digested bovine transferrin and incubated for the indicated time at 20 °C. After the indicated time samples were then centrifuged at 400 g for 10 minutes and supernatants analyzed by Western blot. (A) full-length (approximate molecular weight 66 – 67 kDa) and (B) cleaved bovine transferrin (approximate molecular weight 33 – 37 kDa) with commercial polyclonal rabbit anti-bovine transferrin antibody (Abexxa). n = 3

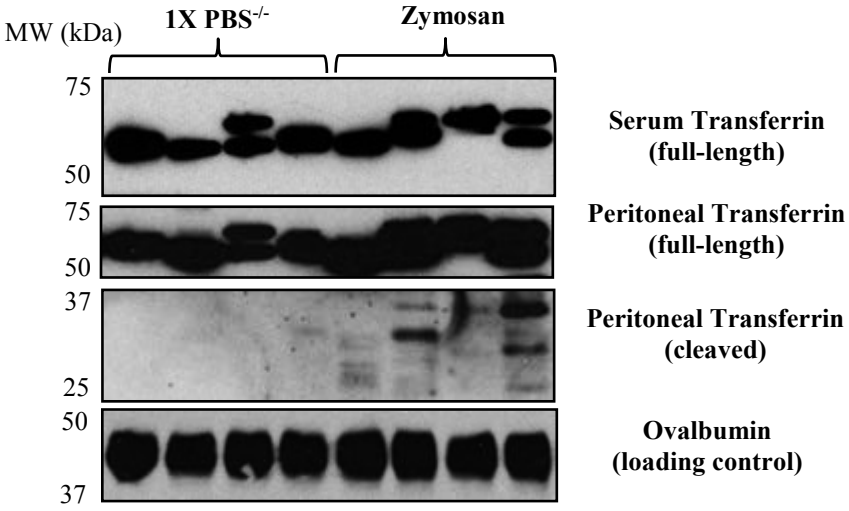



**Figure 4.4 – Goldfish produce transferrin cleavage products at the inflammatory site *in vivo*.** Various samples were collected from goldfish injected with either 1X $PBS^{-/-}$ or 2.5 mg of zymosan 18 hours post injection. Samples were then separated by SDS-PAGE and analysed by Western blot for full-length and cleaved transferrin, approximate molecular weights 65 – 67 kDa and 33 – 37 kDa respectively, using polyclonal rabbit antibody 9AG7. Peritoneal lavage Western blots were stripped and probed for ovalbumin using mouse monoclonal antibody, approximate molecular weight 42 kDa. Ovalbumin was added to 1X $PBS^{-/-}$ at a concentration of 10 $mg \bullet L^{-1}$ prior to peritoneal lavage as an introduced loading control.

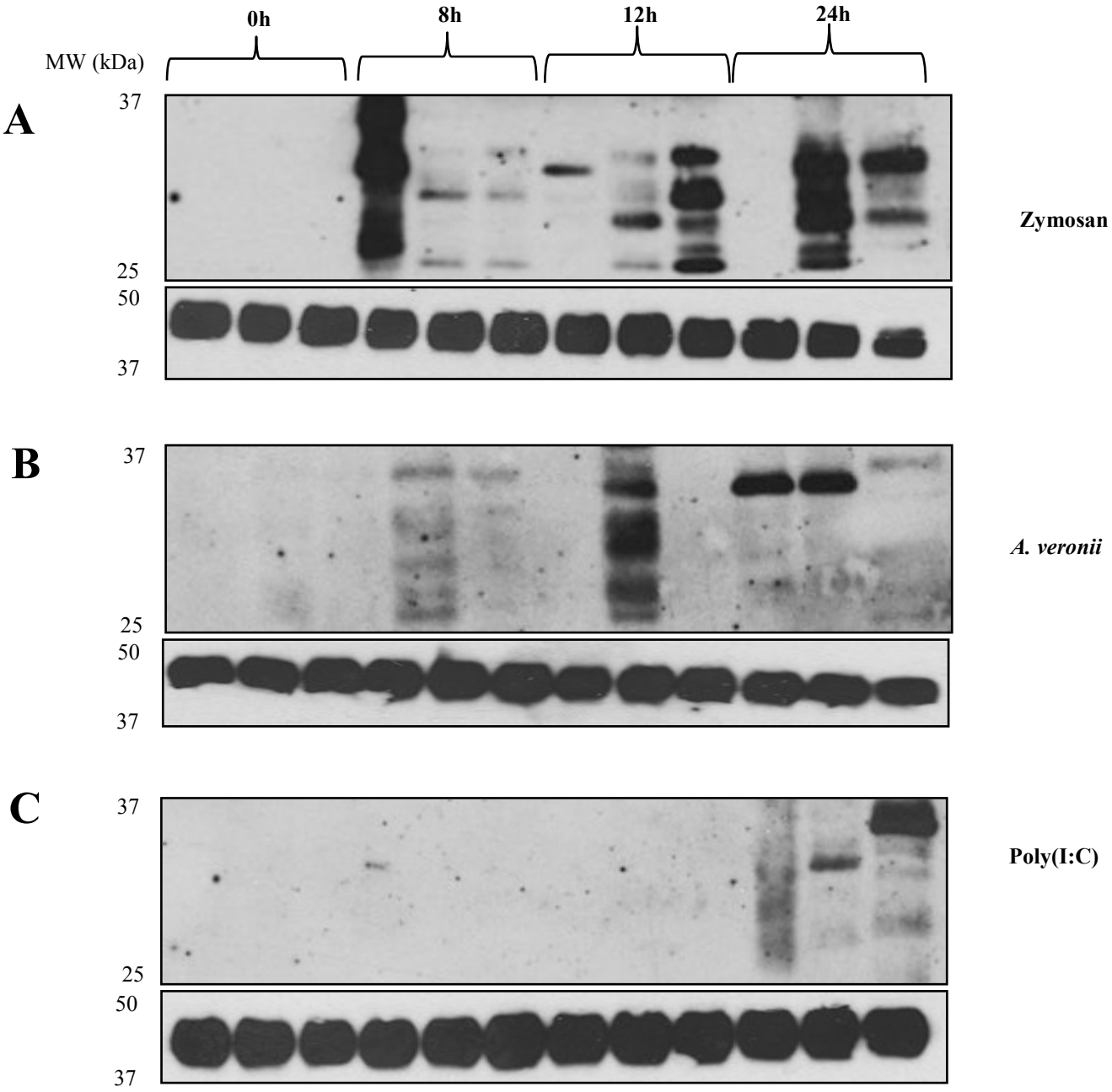



**Figure 4.5 – Goldfish produce transferrin cleavage products with multiple inflammatory stimuli.** Peritoneal lavage samples were collected from goldfish injected with the indicated inflammatory stimuli at the indicated time point, as described in section 2. (A) Goldfish were injected with 2.5 mg of zymosan. (B) Goldfish were injected with 5.0 x $10^6$ C.F.U. of heat-killed *A. veronii*. (C) Goldfish were injected with 200 µg of Poly(I:C). Samples were then separated by SDS-PAGE and analysed by Western blot for cleaved transferrin using 9AG7 as the primary antibody. Western blots were then stripped and probed for ovalbumin as loading control.

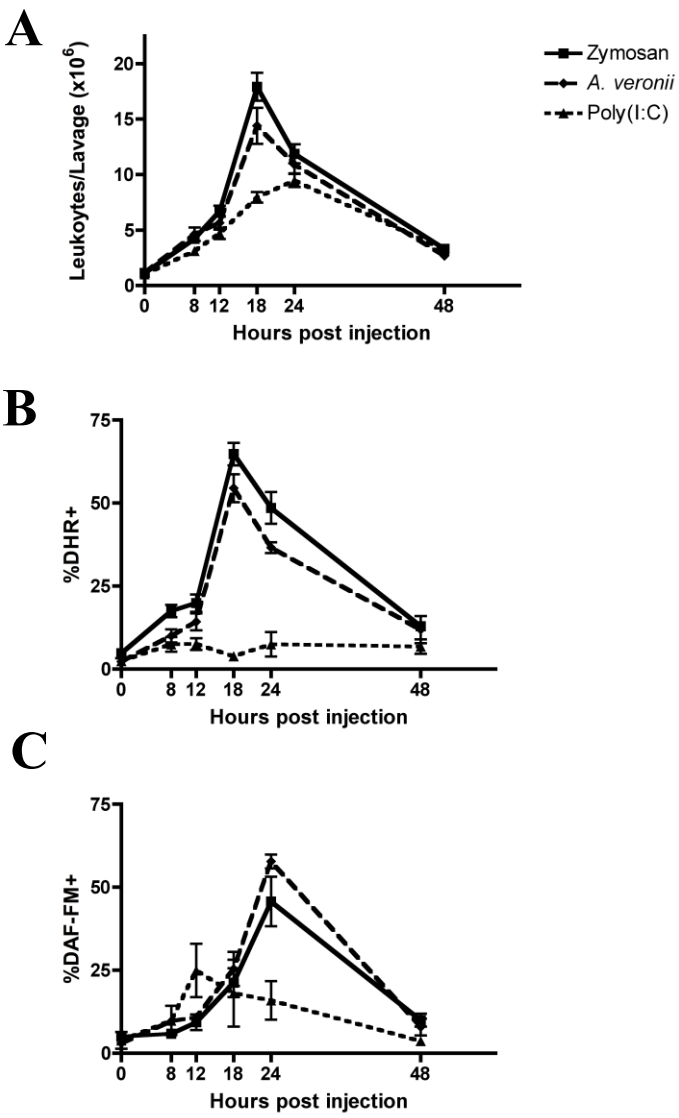



**Figure 4.6. Intraperitoneal injection of pathogens and pathogen mimics induces leucocyte infiltration, respiratory burst, and NO responses**. Goldfish were injected with either 2.5 mg of zymosan, 5.0 x $10^6$ CFU of heat-inactivated *A. veronii*, or 200 µg of Poly(I:C). Cells were harvested by peritoneal lavage at the indicated time point. (A) Leucocytes per lavage at the indicated time point was determined by counting with a haemocytometer. (B) Respiratory burst in isolated leucocytes was determined by DHR staining. (C) NO production or accumulation was determined by DAF-DM diacetate staining. $^{*}P<0.05$ using 2-way analysis of variance and Bonferonni post-hoc test compared to pro-inflammatory peaks, $^{**}P<0.01$ using 2-way analysis of variance and Bonferonni post-hoc test compared to pro-inflammatory peaks.

# Chapter 5: General Discussion and Conclusions

## 5.1 Summary of findings

Acute inflammation is an intricate response that is triggered by noxious stimuli and conditions, such as pathogen invasion and tissue damage. Induction of acute inflammation is triggered by recognition of pathogens or damaged tissue by pattern recognition receptors (PRRs) on tissue-resident macrophages. Binding of stimuli to PRRs leads to the production of inflammatory cytokines, influx of plasma proteins, and recruitment of leucocytes to the inflammatory site. Traditionally, activation of acute inflammation has focused on exogenous stimuli such as PAMPs, virulence factors, allergens, and other foreign bodies (Medzhitov, 2008). However, towards the end of the 20$^{th}$ century Polly Matzinger proposed the danger model of immunity, whereby a host can not only discriminate between self-and non-self molecules, but healthy- and damaged-self molecules (Matzinger, 1994). Damaged tissue arises from injury or infection. During the effector phase of pathogen-induced inflammation professional phagocytes produce toxic effector molecules including ROS, NO, and release toxic granules containing proteases. These effector molecules do not discriminate between pathogens and host tissues, causing collateral damage to host tissues (Serhan and Savill, 2005; Soehnlein and Lindbom, 2010).

### 5.1.1 Summary of data and conclusions

The main focus of my M.Sc. was to characterize the production and contributions of cleaved transferrin products at the inflammatory site *in vivo*. Using an *in vivo* model of self-resolving inflammation in goldfish, *C. auratus*, I was able to show for the first time the endogenous production of cleaved transferrin products *in vivo*. Transferrin cleavage

products have been seen in other systems, specifically found in the bronchoalveolar lavage fluid of cystic fibrosis patients infected with *Pseudomonas aeruginosa* (Britigan et al., 1993), however these cleavage products were likely the result of pathogen degradation rather than endogenous cleavage (Britigan et al., 1992). In this chapter I will provide a summary of my findings, and propose a model of transferrin cleavage and subsequent leucocyte activation in response to pathogen-induced inflammation. I will also propose future experiments to further characterize this model and discuss the relevance of my findings.

I initially found that transferrin cleavage products are present at an inflammatory site *in vivo* (chapter 3). There is a significant volume of work that has characterized transferrin cleavage products using *in vitro* primary macrophage systems in multiple teleost species (Haddad and Belosevic, 2009; Jurecka et al., 2009; Stafford and Belosevic, 2003; Stafford et al., 2001). In these systems transferrin cleavage products were found to induce, and enhance the anti-microbial activity of macrophages resulting in increased NO production, up-regulation of cytokines, and increased leucocyte chemotaxis. The leucocyte-mediated responses (infiltration, respiratory burst, and NO) were extremely consistent within the various pathogens, and pathogen mimics used. However there was significant heterogeneity within transferrin-cleavage product banding patterns. Although my work did not investigate the ability of cleaved transferrin products to induce acute inflammation, this work suggests that cleaved transferrin products are complimentary and not redundant to successful induction of anti-microbial responses. This complimentary would be evident as the presence of cleaved transferrin products were not a pre-requisite for the induction of antimicrobial responses, however given the ability of cleaved

transferrin to induce antimicrobial responses *in vitro* would likely play some role in either resident leucocyte activation, or further activation steps of infiltrating leucocytes. The heterogeneity in transferrin cleavage product banding patterns could be due to a multitude of factors, including inability of the primary antibody used in Western blot to detect smaller transferrin cleavage products, the potential for differential cleavage of the various isoforms of the full-length transferrin, the potential of rapid uptake of transferrin cleavage products by inflammatory leucocytes, and the plethora of extracellular proteases released during inflammation. My results also highlight the possible coordination of both pathogen-induced inflammatory cascades with danger-associated patterns generated during the early stages of acute inflammation.

I then further looked to investigate the contributions of transferrin to acute inflammation *in vivo*. Transferrin has been well characterized as an acute phase protein, being up-regulated by macrophages in response to infection (Cray et al., 2009). This phenomenon is highly conserved with inducible transferrin expression in macrophages seen in insects (Yoshiga et al., 1999; Yoshiga et al., 1997), teleost fish (Stafford and Belosevic, 2003), birds (Xie et al., 2002a), and mammals (Djeha et al., 1995). I further characterized this phenomenon by showing that inflammatory macrophages, but not neutrophils exhibit inducible transferrin expression *in vivo* (chapter 4). Interestingly peritoneal macrophages showed a basal level of constitutive transferrin expression, not seen with *in vitro* primary kidney macrophages cultures (Stafford and Belosevic, 2003). However, *in vivo,* peritoneal macrophages from mice exhibit a basal level of constitutive expression (Djeha et al., 1995). In addition to expression of transferrin by macrophages I show that transferrin cleavage is localized to the inflammatory site, only after pathogen

induced, but not sterile inflammation. This result implies that there is an active-enzyme present only in the inflammatory site that mediates transferrin cleavage. While investigating the source of this enzyme I found that both pro-inflammatory neutrophils, and macrophages have the necessary enzymes to facilitate transferrin cleavage. However the possibly exists that a pro-enzyme is present in the serum that enters the inflammatory site, undergoes a separate cleavage event to become active. There may also be an enzyme produced by the peritoneal epithelium released into the inflammatory site.

Overall, my data, combined with the previous *in vitro characterization* of cleaved transferrin, suggests that cleaved transferrin products may contribute to acute inflammation *in vivo* by being produced during the early stages of inflammation (Haddad and Belosevic, 2009; Stafford and Belosevic, 2003; Stafford et al., 2001). The endogenous production of these cleaved transferrin products may participate in the early autocrine activation events of tissue-resident macrophages (Figure 8.1). The up-regulation of transferrin by macrophages is a primitive adaptation, that combined with the ability of macrophages to secrete cleavage enzymes may promote the propagation of inflammatory events. In this model pathogen binding initially activates resident macrophages. These resident macrophages, express, and potentially produce transferrin locally. This results in an initial increase in transferrin concentration. These macrophages secrete enzymes responsible for cleaving transferrin, resulting in subsequent cleavage to facilitate autocrine and paracrine activation of other macrophages. Later, during the inflammatory response, pro-inflammatory neutrophils enter the site and degranulate releasing more enzymes capable of cleaving transferrin. This further promotes the induction of anti-microbial responses, specifically NO production and secretion of pro-

inflammatory cytokines such as MIP-1γ, RANTES, G-CSF, and IL-6 by macrophages (Haddad and Belosevic, 2009). This model also highlights the potential for a macrophage produced immune transferrin. The production of transferrin by macrophages may temporarily increase the local transferrin concentration at the inflammatory site. There would then be a subsequent decrease in transferrin concentration due to cleavage events, and an increase in holo-transferrin internalization by activated leucocytes. This would be a temporary decrease while transferrin concentration then exhibits a second increase due to serum protein influx later during acute inflammation. Finally, excess transferrin, and any remaining transferrin fragments would be further broken down by intra- and extracellular proteases, eventually being removed from the inflammatory site (Figure 8.1).

In a broader evolutionary context my data suggests that cleaved transferrin products may be present *in vivo* in other organisms. Transferrin molecules are present in a wide range of organisms, which was reviewed during Chapter 1 so will not be discussed here. To date, *in vivo* transferrin cleavage products have only been identified in teleost fish and humans (Britigan et al., 1993). It is noteworthy that the immunostimulatory ability of transferrin cleavage products can be extended to murine macrophage systems (Haddad and Belosevic, 2009). I showed that both neutrophils and macrophages have the ability to induce cleavage of full-length transferrin (chapter 4). Indeed, both transferrin and these phagocytes are highly conserved with homologues present in almost all organisms (Barreda et al., 2016; Browne et al., 2013; Lambert, 2005). Macrophages have been shown to increase expression of transferrin in response in inflammatory stimuli in a wide range of evolutionary lineages (chapter 4) (Djeha et al., 1995; Stafford and Belosevic, 2003; Yoshiga et al., 1999). Both endogenous enzymes (chapter 4), and

pathogens ranging from bacteria to protozoans (Britigan et al., 1993; Ruszczyk et al., 2008) have the ability to facilitate transferrin cleavage. Therefore it is reasonable to speculate that transferrin cleavage products are present in other organisms. Discovering the presence and source of these cleavage products, whether pathogen or endogenous enzyme produced, could help elucidate this potentially primitive mechanism of inflammatory control. I hypothesize that immunostimulatory transferrin cleavage products would also be present in lower evolutionary taxa, specifically arthropods, given their ability to utilize transferrin as an immune molecule, being up-regulated by macrophages (Yoshiga et al., 1997), and being used as an anti-microbial molecule (Yun et al., 2009).

### 5.1.2 Transferrin in iron transport or transferrin as an immune molecule?

The iron-binding capacity of transferrin and transferrin-like molecules has been demonstrated in several organisms, from mammals to organisms as distant as marine algae, for several decades (Lambert, 2012). However the characterization of transferrin as an immune molecule has received significantly less attention in the literature, and the discovery of transferrin's immune functions have been much more recent compared to the iron-binding capability. The requirement for iron- and subsequently iron-binding proteins in all organisms from Archea to humans has been established (Weinberg, 2009). However, evidence of immune-molecules and immune-cells is also evident in the earliest origins of life with Archea and Bacterial biofilms having the capacity to remove dying cells; recycling nutrients and maintaining the clonal biofilm (Hall-Stoodley et al., 2004). Given the necessity of iron for all cell types, and the requirement for iron during the

initial stages of cellular development it seems more likely that transferrin would have evolved iron-binding capacity before it developed immune functions.

## 5.2 Future directions

### 5.2.1 What happens to transferrin cleavage products?

Throughout my Western blot experiments I noticed significant heterogeneity in transferrin cleavage product banding patters and appearance. One potential reason for this is that although the immunological effects of transferrin cleavage products have been well described the fate of transferrin cleavage products is unknown. Recently it has been found that cleaved transferrin products exert their immunological effects through a highly conserved 31 amino acid peptide that is identical between teleost fish and mice (Haddad and Belosevic, 2009). This suggests that the transferrin cleavage products detected by Western blot represent an initial transferrin-cleavage event, however there are subsequent cleavage events that result in peptides capable of macrophage-activation. These smaller cleavage products were not detected by Western blot indicating a more sensitive technique such as mass spectrometry could be used to further characterize the presence of immunostimulatory transferrin cleavage products present at the inflammatory site. Indeed, using mass spectrometry might also help elucidate the phenomenon of differential capacity of transferrin isoforms to induce anti-microbial effects (Jurecka et al., 2009).

In addition to the generation of smaller transferrin breakdown products the cell-receptor, or receptors, have not been elucidated that bind transferrin cleavage products. Although I was able to show that peritoneal leucocytes have the capacity to remove transferrin cleavage products from the inflammatory site (Chapter 4) it is not known

whether they remove transferrin cleavage products by facilitating extracellular degradation of these products, or internalizing these products and degrading them through the intracellular proteasome. To investigate the down-stream consequences that await transferrin cleavage products I would need to generate recombinant-tagged immunoreactive transferrin-peptides. This would need to be done with something quite small, such as a V5 epitope that would not interfere with binding. I could then separate out leucocyte populations and examine banding patterns of these populations using Western blotting with a V5-epitope primary antibody. To ensure effective binding of the anti-microbial peptide I would have to monitor NO responses, and cytokine production. I predict that in analysing sub-populations I would see significant association of transferrin-peptide with macrophages. I may also see association of transferrin-peptide with neutrophils and/or lymphocytes as it has also been noted that these two sub-populations also express a suite of PRR's, capable of recognizing DAMPs (Zhang and Mosser, 2008). However this transferrin peptide may also bind briefly to its receptor, exerting its anti-microbial effect, and then may be immediately degraded extracellular which would result it no detection of banding by Western blot. Therefore supernatants from transferrin-peptide and leucocytes would also be analysed for smaller bands containing the epitope tag.

### 5.2.2 Do transferrin cleavage products have a direct effect on neutrophils?

Effects of transferrin cleavage products on macrophages have been well described. These cleavage products likely act on macrophages *in vivo* causing production of pro-inflammatory cytokines that act on neutrophils thereby indirectly activating neutrophils. However the direct effects of transferrin cleavage products on neutrophils have not been

investigated, despite neutrophils playing a critical role during acute inflammation. Indeed, in zymosan induced peritonitis neutrophils are the primary infiltrating cell type, being the first leucocyte to see significant influx into the inflammatory site, and comprising nearly 50% of the infiltrating leucocyte populations (Havixbeck et al., 2016; Rieger et al., 2012; Rieger et al., 2015). I briefly investigated the role of neutrophils in generating transferrin cleavage products (Chapter 4), and showed that pro-inflammatory neutrophils posses enzymes necessary to facilitate initial transferrin cleavage events. To investigate the direct effects of transferrin on neutrophils I could use a neutrophil culture technique previously reported by Katzenback and Belosevic (2009) and treat these cells with the immunoreactive transferrin-peptide (Haddad and Belosevic, 2009). I could then monitor the ability of these neutrophils to induce respiratory burst, produce pro-inflammatory cytokines, and undergo degranulation. Given the pro-inflammatory effects of transferrin cleavage products noted on macrophages I predict that transferrin cleavage products would also be able to act directly on neutrophils to further promote pro-inflammatory responses.

## 5.3 Relevance

### 5.3.1 To basic biology

Transferrin is critical to a number of biological processes, including: iron metabolism, host sequestration of iron from pathogens, cell maturation, and during inflammation. By studying the contributions of transferrin to acute inflammation we will gain a better understanding of this, potentially primitive, mechanism of immune activation and regulation. Further it will shed light on the evolutionary development of

the ability of damaged-self molecules to tailor the immune response to ensure effective pathogen clearance, while maintaining host-tissue integrity.

### 5.3.2 To medicine

To date there is significant work in manipulating transferrin for its role in iron metabolism. For example, injection of transferrin has been used to abrogate the effects of iron overload diseases (Lie et al., 2009). In addition to manipulating transferrin for use in iron metabolism diseases, transferrin has been used for targeted delivery of drugs, proteins, and even genes to combat various diseases (Qian et al., 2002). Given that transferrin cleavage products are commonly found in the lungs of cystic fibrosis patients (Britigan et al., 1993) investigating the role of transferrin cleavage products as an immune activator could have other broad medical applications.

## 5.4 Summary

The main objective of my M.Sc. was to investigate the regulation of cleaved transferrin products during acute inflammation. I specifically focused on the ability of cleaved transferrin products during the activation stage of acute inflammation, investigating a) are we able to use transferrin as a broader marker for the early stages of the acute inflammatory response and b) if transferrin cleavage products play a role during acute inflammation *in vivo*. My research has shown for the first time the endogenous production of cleaved transferrin products *in vivo*. The identification of transferrin cleavage products early in pathogen induced, but not sterile, inflammation suggests that transferrin cleavage products may participate during the early activation events of acute inflammation. Indeed, given the presence of transferrin, and transferrin-like cells present

in almost all organisms cleaved transferrin products may be a highly conserved activation mechanisms of the innate immune system.

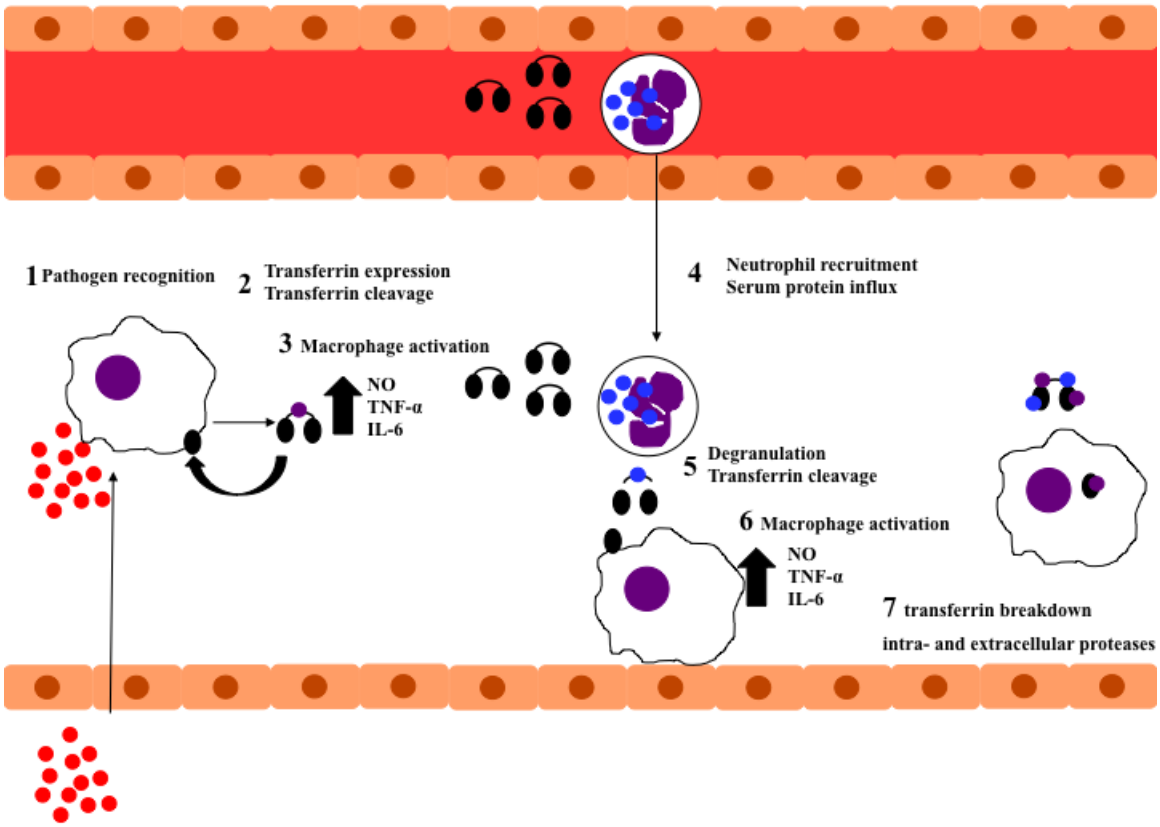



**Figure 5.1 – Proposed model of macrophage activation by endogenous production of cleaved transferrin products.** Pathogen invasion results in increased expression of transferrin, and subsequent cleavage by resident macrophages. Later in the inflammatory response there is neutrophil and serum protein influx. Neutrophils degranulate, further cleaving full-length transferrin and activating macrophages.

# References

Adams, D.H., Hathaway, M., Shaw, J., Burnett, D., Elias, E., Strain, A.J., 1991. Transforming growth factor-beta induces human T lymphocyte migration in vitro. The Journal of Immunology 147, 609-612.
Aisen P Fau - Leibman, A., Leibman A Fau - Zweier, J., Zweier, J., 1978. Stoichiometric and site characteristics of the binding of iron to human transferrin.
Alexopoulou, L., Holt, A.C., Medzhitov, R., Flavell, R.A., 2001. Recognition of double-stranded RNA and activation of NF-κB by Toll-like receptor 3. Nature 413, 732-738.
Allen, J.B., Manthey, C.L., Hand, A.R., Ohura, K., Ellingsworth, L., Wahl, S., 1990. Rapid onset synovial inflammation and hyperplasia induced by transforming growth factor beta. The Journal of experimental medicine 171, 231-247.
Antipa, R., Amend, D.F., 1977. Immunization of Pacific salmon: comparison of intraperitoneal injection and hyperosmotic infiltration of Vibrio anguillarum and Aeromonas salmonicida bacterins. Journal of the Fisheries Board of Canada 34, 203-208.
Baker, E.N., Baker, H.M., Kidd, R.D., 2002. Lactoferrin and transferrin: functional variations on a common structural framework. Biochemistry and cell biology 80, 27-34.
Barreda, D. R., Neely, H. R., Flajnik, M. F. 2016. Evolution of myeloid cells. Microbiology spectrum 4, MCHD-0007-2015.
Baumann, H., Gauldie, J., 1994. The acute phase response. Immunology Today 15, 74-80.
Bayne, C.J., Gerwick, L., 2001. The acute phase response and innate immunity of fish. Developmental & Comparative Immunology 25, 725-743.
Berkman, N., John, M., Roesems, G., Jose, P.J., Barnes, P.J., Chung, K.F., 1995. Inhibition of macrophage inflammatory protein-1 alpha expression by IL-10. Differential sensitivities in human blood monocytes and alveolar macrophages. The Journal of Immunology 155, 4412-4418.
Bogdan, C., 2001. Nitric oxide and the immune response. Nature immunology 2, 907-916.
Boltana, S., Donate, C., Goetz, F.W., MacKenzie, S., Balasch, J.C., 2009. Characterization and expression of NADPH oxidase in LPS-, poly (I: C)-and zymosan-stimulated trout (Oncorhynchus mykiss W.) macrophages. Fish & shellfish immunology 26, 651-661.
Bournazou, I., Pound, J.D., Duffin, R., Bournazos, S., Melville, L.A., Brown, S.B., Rossi, A.G., Gregory, C.D., 2009. Apoptotic human cells inhibit migration of granulocytes via release of lactoferrin. The Journal of clinical investigation 119, 20-32.
Breton-Gorius, J., Mason, D.Y., Buriot, D., Vilde, J.L., Griscelli, C., 1980. Lactoferrin deficiency as a consequence of a lack of specific granules in neutrophils from a patient with recurrent infections. Detection by immunoperoxidase staining for lactoferrin and cytochemical electron microscopy. The American Journal of Pathology 99, 413-428.
Britigan, B.E., Hayek, M.B., Doebbeling, B.N., Fick, R.B., Jr., 1993. Transferrin and lactoferrin undergo proteolytic cleavage in the Pseudomonas aeruginosa-infected lungs of patients with cystic fibrosis. Infection and immunity 61, 5049-5055.
Britigan, B.E., Roeder, T.L., Rasmussen, G.T., Shasby, D.M., McCormick, M.L., Cox, C.D., 1992. Interaction of the Pseudomonas aeruginosa secretory products pyocyanin and pyochelin generates hydroxyl radical and causes synergistic damage to endothelial cells.

Implications for Pseudomonas-associated tissue injury. Journal of Clinical Investigation 90, 2187-2196.
Broeg, K., Steinhagen, D., 2011. Attack and defense: reactive oxygen and nitrogen species in teleost fish immune response and the coevolved evasion of microbes and parasites. Oxidative Stress in Aquatic Ecosystems, 247-260.
Brooks, J.M., Wessel, G.M., 2002. The major yolk protein in sea urchins is a transferrin-like, iron binding protein. Developmental biology 245, 1-12.
Browne, N., Heelan, M., Kavanagh, K. 2013. An analysis of the structural and functional similarities of insect hemocytes and mammalin phagocytes. Virulence 4, 597-603.
Buckley, C.D., Pilling, D., Lord, J.M., Akbar, A.N., Scheel-Toellner, D., Salmon, M., 2001. Fibroblasts regulate the switch from acute resolving to chronic persistent inflammation. Trends in immunology 22, 199-204.
Bullen, J., Griffiths, E., Edmiston, C.E., 1999. Iron and infection: molecular, physiological and clinical aspects. Shock 12, 410.
Byrd, T.F., Horwitz, M.A., 1989. Interferon gamma-activated human monocytes downregulate transferrin receptors and inhibit the intracellular multiplication of Legionella pneumophila by limiting the availability of iron. Journal of Clinical Investigation 83, 1457.
Campagnari, A.A., Shanks, K.L., Dyer, D.W., 1994. Growth of Moraxella catarrhalis with human transferrin and lactoferrin: expression of iron-repressible proteins without siderophore production. Infection and immunity 62, 4909-4914.
Capsoni, F., Minonzio, F., Ongari, A.M., Carbonelli, V., Galli, A., Zanussi, C., 1995. IL-10 up-regulates human monocyte phagocytosis in the presence of IL-4 and IFN-gamma. Journal of leucocyte biology 58, 351-358.
Cassatella, M.A., Gasperini, S., Calzetti, F., Bertagnin, A., Luster, A.D., McDonald, P.P., 1997. Regulated production of the interferon- γ- inducible protein− 10 (IP- 10) chemokine by human neutrophils. European journal of immunology 27, 111-115.
Cavill, I., 2002. Erythropoiesis and iron. Best Practice & Research Clinical Haematology 15, 399-409.
Chandran, M., Aruna, B., Logambal, S., Michael, R.D., 2002. Immunisation of Indian major carps against Aeromonas hydrophila by intraperitoneal injection. Fish & shellfish immunology 13, 1-9.
Choe, Y.H., Oh, Y.J., Lee, N.G., Imoto, I., Adachi, Y., Toyoda, N., Gabazza, E.C., 2003. Lactoferrin sequestration and its contribution to iron-deficiency anemia in Helicobacter pylori-infected gastric mucosa. Journal of Gastroenterology and Hepatology 18, 980-985.
Cornelissen, C.N., 2003. Transferrin-iron uptake by Gram-negative bacteria, Front Biosci, pp. d836-847.
Cray, C., Zaias, J., Altman, N.H., 2009. Acute phase response in animals: a review. Comparative medicine 59, 517-526.
Cui, W., Morrison, D.C., Silverstein, R., 2000. Differential tumor necrosis factor alpha expression and release from peritoneal mouse macrophages in vitro in response to proliferating gram-positive versus gram-negative bacteria. Infection and immunity 68, 4422-4429.
Dautry-Varsat, A., Ciechanover, A., Lodish, H.F., 1983. pH and the recycling of transferrin during receptor-mediated endocytosis. Proceedings of the National Academy of Sciences of the United States of America 80, 2258-2262.

de Waal Malefyt, R., Abrams, J., Bennett, B., Figdor, C., De Vries, J., 1991. IL-10 inhibits cytokine synthesis by human monocytes: an autocrine role of IL-10 produced by monocytes. J. Exp. Med 250, 1302-1305.
Decker, T., Stockinger, S., Karaghiosoff, M., Müller, M., Kovarik, P., 2002. IFNs and STATs in innate immunity to microorganisms. The Journal of clinical investigation 109, 1271-1277.
Dillon, S., Agrawal, S., Banerjee, K., Letterio, J., Denning, T.L., Oswald-Richter, K., Kasprowicz, D.J., Kellar, K., Pare, J., van Dyke, T., 2006. Yeast zymosan, a stimulus for TLR2 and dectin-1, induces regulatory antigen-presenting cells and immunological tolerance. The Journal of clinical investigation 116, 916-928.
Dinarello, C.A., 2011. Interleukin-1 in the pathogenesis and treatment of inflammatory diseases. Blood 117, 3720-3732.
Ding, A.H., Nathan, C.F., Stuehr, D.J., 1988. Release of reactive nitrogen intermediates and reactive oxygen intermediates from mouse peritoneal macrophages. Comparison of activating cytokines and evidence for independent production. The Journal of Immunology 141, 2407-2412.
Djeha, A., Brock, J.H., 1992. Effect of transferrin, lactoferrin and chelated iron on human T-lymphocytes. British Journal of Haematology 80, 235-241.
Djeha, A., Perez-Arellano, J.L., Hayes, S.L., Oria, R., Simpson, R.J., Raja, K.B., Brock, J.H., 1995. Cytokine-mediated regulation of transferrin synthesis in mouse macrophages and human T lymphocytes. Blood 85, 1036-1042.
Doherty, C.P., 2007. Host-pathogen interactions: the role of iron. The Journal of nutrition 137, 1341-1344.
Doherty, N.S., Poubelle, P., Borgeat, P., Beaver, T.H., Westrich, G.L., Schrader, N.L., 1985. Intraperitoneal injection of zymosan in mice induces pain, inflammation and the synthesis of peptidoleukotrienes and prostaglandin E2. Prostaglandins 30, 769-789.
Dunkov, B., Georgieva, T., 2006. Insect iron binding proteins: insights from the genomes. Insect biochemistry and molecular biology 36, 300-309.
Dunn, L.L., Sekyere, E.O., Suryo Rahmanto, Y., Richardson, D.R., 2006. The function of melanotransferrin: a role in melanoma cell proliferation and tumorigenesis. Carcinogenesis 27, 2157-2169.
Ellis, A.E., 2001. Innate host defense mechanisms of fish against viruses and bacteria. Developmental & Comparative Immunology 25, 827-839.
Esmann, L., Idel, C., Sarkar, A., Hellberg, L., Behnen, M., Möller, S., van Zandbergen, G., Klinger, M., Köhl, J., Bussmeyer, U., 2010. Phagocytosis of apoptotic cells by neutrophil granulocytes: diminished proinflammatory neutrophil functions in the presence of apoptotic cells. The journal of immunology 184, 391-400.
Evans, W.H., Wilson, S.M., Bednarek, J.M., Peterson, E.A., Knight, R.D., Mage, M.G., McHugh, L., 1989. Evidence for a factor in normal human serum that induces human neutrophilic granulocyte end-stage maturation in vitro. Leukemia research 13, 673-682.
Evans, W.H., Wilson, S.M., Mage, M.G., 1986. Transferrin induces maturation of neutrophil granulocyte precursors in vitro. Leukemia research 10, 429-436.
Farnaud, S., Evans, R.W., 2003. Lactoferrin—a multifunctional protein with antimicrobial properties. Molecular immunology 40, 395-405.
Faurschou, M., Borregaard, N., 2003. Neutrophil granules and secretory vesicles in inflammation. Microbes and Infection 5, 1317-1327.

Feelders, Vreugdenhil , Eggermont , Kuiper, K., Van, E., Swaak 1998. Regulation of iron metabolism in the acute-phase response: interferon γ and tumour necrosis factor α induce hypoferraemia, ferritin production and a decrease in circulating transferrin receptors in cancer patients. European Journal of Clinical Investigation 28, 520-527.
Fiorentino, D.F., Zlotnik, A., Mosmann, T., Howard, M., O'garra, A., 1991. IL-10 inhibits cytokine production by activated macrophages. The Journal of Immunology 147, 3815-3822.
Fisher, M., Gokhman, I., Pick, U., Zamir, A., 1997. A structurally novel transferrin-like protein accumulates in the plasma membrane of the unicellular green alga Dunaliella salina grown in high salinities. The Journal of biological chemistry 272, 1565-1570.
Fisher, M., Zamir, A., Pick, U., 1998. Iron uptake by the halotolerant alga Dunaliella is mediated by a plasma membrane transferrin. The Journal of biological chemistry 273, 17553-17558.
Forlenza, M., Scharsack, J.P., Kachamakova, N.M., Taverne-Thiele, A.J., Rombout, J.H., Wiegertjes, G.F., 2008. Differential contribution of neutrophilic granulocytes and macrophages to nitrosative stress in a host–parasite animal model. Molecular immunology 45, 3178-3189.
Frucht, D.M., Fukao, T., Bogdan, C., Schindler, H., O'Shea, J.J., Koyasu, S., 2001. IFN-γ production by antigen-presenting cells: mechanisms emerge. Trends in immunology 22, 556-560.
García-Montoya, I.A., Cendón, T.S., Arévalo-Gallegos, S., Rascón-Cruz, Q., 2012. Lactoferrin a multiple bioactive protein: an overview. Biochimica et Biophysica Acta (BBA)-General Subjects 1820, 226-236.
Geiser, D.L., Zhang, D., Winzerling, J.J., 2006. Secreted ferritin: mosquito defense against iron overload? Insect biochemistry and molecular biology 36, 177-187.
Gerlach, G.-F., Klashinsky, S., Anderson, C., Potter, A., Willson, P., 1992. Characterization of two genes encoding distinct transferrin-binding proteins in different Actinobacillus pleuropneumoniae isolates. Infection and immunity 60, 3253-3261.
Gewirtz, A. T., Navas, T. A., Lyons, S., Godowski, P. J., Madara, J. L. 2001. Cutting edge: bacterial flagellin activates basolaterally expressed TLR5 to induce epithelial proinflammatory gene expression. Journal of Immunology 167, 1882-1885.
Giansanti, F., Leboffe, L., Pitari, G., Ippoliti, R., Antonini, G., 2012. Physiological roles of ovotransferrin. Biochimica et biophysica acta 1820, 218-225.
Gkouvatsos, K., Papanikolaou, G., Pantopoulos, K., 2012. Regulation of iron transport and the role of transferrin. Biochimica et Biophysica Acta (BBA)-General Subjects 1820, 188-202.
Gruys, E., Toussaint, M., Niewold, T., Koopmans, S., 2005. Acute phase reaction and acute phase proteins. J Zhejiang Univ Sci B 6, 1045-1056.
Haas, J., Roth, S., Arnold, K., Kiefer, F. Schmidt, T., Bordoli, L., Schwede, T. 2013. The protein model portal - a compreshensive resocurse for protein structure and model information.
Haddad, G., Belosevic, M., 2009. Transferrin-derived synthetic peptide induces highly conserved pro-inflammatory responses of macrophages. Molecular immunology 46, 576-586.
Hall-Stoodley, L. Costerton J. W., Sottdley, P. 2004. Bacterial biofilms: from the ntrual environment to infectious diseases. Nature reviews microbiology 2, 95-108.

Hamill, R. J., Vann, J. M., Proctor, R. A. 1986. Phagocytosis of Staphylococcus aureus by culture bovine aortic endothelial cells: model for postahderence events in endocascular infections. Infection and Immunity 54, 833-836.
Hamilton, T.A., Gray, P.W., Adams, D.O., 1984. Expression of the transferrin receptor on murine peritoneal macrophages is modulated by in vitro treatment with interferon gamma. Cellular immunology 89, 478-488.
Harden, L.M., du Plessis, I., Poole, S., Laburn, H.P., 2008. Interleukin (IL)-6 and IL-1β act synergistically within the brain to induce sickness behavior and fever in rats. Brain, behavior, and immunity 22, 838-849.
Harding, C., Heuser, J., Stahl, P., 1983. Receptor-mediated endocytosis of transferrin and recycling of the transferrin receptor in rat reticulocytes. The Journal of cell biology 97, 329-339.
Harris, S.G., Padilla, J., Koumas, L., Ray, D., Phipps, R.P., 2002. Prostaglandins as modulators of immunity. Trends in immunology 23, 144-150.
Harrison, P.M., Arosio, P., 1996. The ferritins: molecular properties, iron storage function and cellular regulation. Biochimica et Biophysica Acta (BBA) - Bioenergetics 1275, 161-203.
Havixbeck, J.J., Rieger, A.M., Wong, M.E., Hodgkinson, J.W., Barreda, D.R., 2016. Neutrophil contributions to the induction and regulation of the acute inflammatory response in teleost fish. Journal of leucocyte biology 99, 241-252.
Hayley, M., Sun, M., Merschrod, E.F., Davis, P.J., Robinson, J.J., 2008. Biochemical analysis of the interaction of calcium with toposome: a major protein component of the sea urchin egg and embryo. Journal of cellular biochemistry 103, 1464-1471.
Herrington, D., Sparling, P., 1985. Haemophilus influenzae can use human transferrin as a sole source for required iron. Infection and immunity 48, 248-251.
Hesse, D.G., Tracey, K.J., Fong, Y., Manogue, K.R., Palladino, M.A., Cerami, A., Shires, G.T., Lowry, S.F., 1988. Cytokine appearance in human endotoxemia and primate bacteremia. Surg Gynecol Obstet 166, 147-153.
Heyworth, P.G., Cross, A.R., Curnutte, J.T., 2003. Chronic granulomatous disease. Current opinion in immunology 15, 578-584.
Hissen, A., Chow, J., Pinto, L., Moore, M., 2004. Survival of Aspergillus fumigatus in serum involves removal of iron from transferrin: the role of siderophores. Infection and immunity 72, 1402-1408.
Huebers, H., Huebers, E., Finch, C., Webb, B., Truman, J., Riddiford, L., Martin, A., Massover, W., 1988. Iron binding proteins and their roles in the tobacco hornworm, Manduca sexta (L.). Journal of Comparative Physiology B 158, 291-300.
Ibrahim, H.R., Iwamori, E., Sugimoto, Y., Aoki, T., 1998. Identification of a distinct antibacterial domain within the N-lobe of ovotransferrin. Biochimica et Biophysica Acta (BBA)-Molecular Cell Research 1401, 289-303.
Ibrahim, H.R., Sugimoto, Y., Aoki, T., 2000. Ovotransferrin antimicrobial peptide (OTAP-92) kills bacteria through a membrane damage mechanism. Biochimica et Biophysica Acta (BBA)-General Subjects 1523, 196-205.
Janssens, S., Beyaert, R., 2002. A universal role for MyD88 in TLR/IL-1R-mediated signaling. Trends in biochemical sciences 27, 474-482.
Johnson, E.E., Wessling-Resnick, M., 2012. Iron metabolism and the innate immune response to infection. Microbes and Infection 14, 207-216.

Jurecka, P., Irnazarow, I., Stafford, J.L., Ruszczyk, A., Taverne, N., Belosevic, M., Savelkoul, H.F.J., Wiegertjes, G.F., 2009. The induction of nitric oxide response of carp macrophages by transferrin is influenced by the allelic diversity of the molecule. Fish & shellfish immunology 26, 632-638.
Kasama, T., Strieter, R.M., Lukacs, N.W., Burdick, M.D., Kunkel, S.L., 1994. Regulation of neutrophil-derived chemokine expression by IL-10. The Journal of Immunology 152, 3559-3569.
Katzenback, B.A., Belosevic, M., 2009. Isolation and functional characterization of neutrophil-like cells, from goldfish (Carassius auratus L.) kidney. Developmental & Comparative Immunology 33, 601-611.
Kawai, T., Akira, S., 2010. The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. Nature immunology 11, 373-384.
Kehl-Fie, T.E., Skaar, E.P., 2010. Nutritional immunity beyond iron: a role for manganese and zinc. Current opinion in chemical biology 14, 218-224.
Kim, B.Y., Lee, K.S., Choo, Y.M., Kim, I., Je, Y.H., Woo, S.D., Lee, S.M., Park, H.C., Sohn, H.D., Jin, B.R., 2008. Insect transferrin functions as an antioxidant protein in a beetle larva. Comparative biochemistry and physiology. Part B, Biochemistry & molecular biology 150, 161-169.
Kopydlowski, K.M., Salkowski, C.A., Cody, M.J., van Rooijen, N., Major, J., Hamilton, T.A., Vogel, S.N., 1999. Regulation of macrophage chemokine expression by lipopolysaccharide in vitro and in vivo. The Journal of Immunology 163, 1537-1544.
Korhonen, R., Lahti, A., Kankaanranta, H., Moilanen, E., 2005. Nitric oxide production and signaling in inflammation. Current Drug Targets-Inflammation & Allergy 4, 471-479.
Kovacevic, N., Hagen, M.O., Xie, J., Belosevic, M., 2015. The analysis of the acute phase response during the course of Trypanosoma carassii infection in the goldfish (Carassius auratus L.). Developmental & Comparative Immunology 53, 112-122.
Kucharski, R., Maleszka, R., 2003. Transcriptional profiling reveals multifunctional roles for transferrin in the honeybee, Apis mellifera. Journal of insect science (Online) 3, 27.
Laichalk, L.L., Danforth, J.M., Standiford, T.J., 1996. Interleukin-10 inhibits neutrophil phagocytic and bactericidal activity. FEMS Immunology & Medical Microbiology 15, 181-187.
Lambert, L.A., 2012. Molecular evolution of the transferrin family and associated receptors. Biochimica et biophysica acta 1820, 244-255.
Lambert, L.A., Perri, H., Meehan, T.J., 2005. Evolution of duplications in the transferrin family of proteins. Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology 140, 11-25.
Laskey, J., Webb, I., Schulman, H.M., Ponka, P., 1988. Evidence that transferrin supports cell proliferation by supplying iron for DNA synthesis. Experimental Cell Research 176, 87-95.
Le, N.T.V., Richardson, D.R., 2002. The role of iron in cell cycle progression and the proliferation of neoplastic cells. Biochimica et Biophysica Acta (BBA) - Reviews on Cancer 1603, 31-46.
Leiro, J., Iglesias, R., Parama, A., Sanmartín, M., Ubeira, F., 2001. Effect of Tetramicra brevifilum (Microspora) infection on respiratory-burst responses of turbot (Scophthalmus maximus L.) phagocytes. Fish & shellfish immunology 11, 639-652.

Li, H., Rybicki, A. C., Suzuka, S. M., von Bonsdorff, L., Breuer, W., Hall, C. B., Cabantchik, Z. I., Bouhassira, E. E., Fabry, M. E., Ginzburg Y. Z. 2009. Transferrin therapy ameliorates disease in β-thalassemic mice. Nature medicine 16, 177-183.
Li, M., Carpio, D. F., Zheng, Y., Bruzzo, P., Singh, V., Ouaaz, F., Medzhitov, R. M., Beg, A. A. 2001. An essential role of the NF-κB/Toll-like receptor pathway in induction of inflammatory and tissue repair gene expression by necrotic cells.
Li, Y., Moczydlowski, E., 1991. Purification and partial sequencing of saxiphilin, a saxitoxin-binding protein from the bullfrog, reveals homology to transferrin. The Journal of biological chemistry 266, 15481-15487.
Lieu, P.T., Heiskala, M., Peterson, P.A., Yang, Y., 2001. The roles of iron in health and disease. Molecular aspects of medicine 22, 1-87.
Lima, M.F., Villalta, F., 1990. Trypanosoma cruzi receptors for human transferrin and their role. Molecular and biochemical parasitology 38, 245-252.
Lincoln, J., Hoyle, C.H., Burnstock, G., 1997. Nitric oxide in health and disease. Cambridge University Press.
Llewellyn, L.E., Bell, P.M., Moczydlowski, E.G., 1997. Phylogenetic survey of soluble saxitoxin-binding activity in pursuit of the function and molecular evolution of saxiphilin, a relative of transferrin. Proceedings. Biological sciences / The Royal Society 264, 891-902.
Lohmann-Matthes, M., Lüttig, B., Hockertz, S., 1991. Involvement of membrane-associated TNF in the killing of Leishmania donovani parasites by macrophages. Behring Institute Mitteilungen, 125-132.
Macedo, M.F., De Sousa, M., Ned, R.M., Mascarenhas, C., Andrews, N.C., Correia-Neves, M., 2004. Transferrin is required for early T-cell differentiation. Immunology 112, 543-549.
Macedo, M.F., Sousa, M.d., 2008. Transferrin and the transferrin receptor: of magic bullets and other concerns. Inflammation & Allergy-Drug Targets (Formerly Current Drug Targets-Inflammation & Allergy) 7, 41-52.
Marfaing-Koka, A., Maravic, M., Humbert, M., Galanaud, P., Emllie, D., 1996. Contrasting effects of IL-4, IL-10 and corticosteroids on RANTES production by human monocytes. International immunology 8, 1587-1594.
Martín-Fontecha, A., Thomsen, L.L., Brett, S., Gerard, C., Lipp, M., Lanzavecchia, A., Sallusto, F., 2004. Induced recruitment of NK cells to lymph nodes provides IFN-γ for TH1 priming. Nature immunology 5, 1260-1265.
Martinez, F.O., Sica, A., Mantovani, A., Locati, M., 2007. Macrophage activation and polarization. Frontiers in bioscience: a journal and virtual library 13, 453-461.
Matzinger, P., 1994. Tolerance, danger, and the extended family. Annual review of immunology 12, 991-1045.
Mayumi, M., Takeda, Y., Hoshiko, M., Serada, K., Murata, M., Moritomo, T., Takizawa, F., Kobayashi, I., Araki, K., Nakanishi, T., 2008. Characterization of teleost phagocyte NADPH oxidase: molecular cloning and expression analysis of carp (Cyprinus carpio) phagocyte NADPH oxidase. Molecular immunology 45, 1720-1731.
Medzhitov, R., 2001. Toll-like receptors and innate immunity. Nature Reviews Immunology 1, 135-145.
Medzhitov, R., 2008. Origin and physiological roles of inflammation. Nature 454, 428-435.

Metz- Boutigue, M.H., Jollés, J., Mazurier, J., Schoentgen, F., Legrand, D., Spik, G., Montreuil, J., Jollès, P., 1984. Human lactotransferrin: amino acid sequence and structural comparisons with other transferrins. European Journal of Biochemistry 145, 659-676.
Mickelsen, P.A., Sparling, P.F., 1981. Ability of Neisseria gonorrhoeae, Neisseria meningitidis, and commensal Neisseria species to obtain iron from transferrin and iron compounds. Infection and immunity 33, 555-564.
Mikawa, N., Hirono, I., Aoki, T., 1996. Structure of medaka transferrin gene and its 5'-flanking region. Mol Mar Biol Biotechnol 5, 225-229.
Ming, W.J., Bersani, L., Mantovani, A., 1987. Tumor necrosis factor is chemotactic for monocytes and polymorphonuclear leucocytes. The Journal of Immunology 138, 1469-1474.
Møller, D., 1970. Transferrin Polymorphism in Atlantic Salmon (Salmo salar). Journal of the Fisheries Research Board of Canada 27, 1617-1625.
Morabito, M.A., Moczydlowski, E., 1995. Molecular cloning of bullfrog saxiphilin: a unique relative of the transferrin family that binds saxitoxin. Proc Natl Acad Sci U S A 92, 6651.
Murayama, E., Okuno, A., Ohira, T., Takagi, Y., Nagasawa, H., 2000. Molecular cloning and expression of an otolith matrix protein cDNA from the rainbow trout, Oncorhynchus mykiss. Comparative biochemistry and physiology. Part B, Biochemistry & molecular biology 126, 511-520.
Nathan, C., Xie, Q.-w., 1994. Nitric oxide synthases: roles, tolls, and controls. Cell 78, 915-918.
Neumann, N.F., 1999. Regulation of antimicrobial mechanisms of macrophages of the goldfish (Carassius auratus).
Neumann, N.F., Barreda, D.R., Belosevic, M., 2000. Generation and functional analysis of distinct macrophage sub-populations from goldfish (Carassius auratus L.) kidney leucocyte cultures. Fish & Shellfish Immunology 10, 1-20.
Newman, S.L., 1999. Macrophages in host defense against Histoplasma capsulatum. Trends in microbiology 7, 67-71.
Noll, H., Alcedo, J., Daube, M., Frei, E., Schiltz, E., Hunt, J., Humphries, T., Matranga, V., Hochstrasser, M., Aebersold, R., Lee, H., Noll, M., 2007. The toposome, essential for sea urchin cell adhesion and development, is a modified iron-less calcium-binding transferrin. Developmental biology 310, 54-70.
Oppenheimer-Marks, N., Ziff, M., 1988. Migration of lymphocytes through endothelial cell monolayers: augmentation by interferon-γ. Cellular immunology 114, 307-323.
Paz, Y., Katz, A., Pick, U., 2007. A multicopper ferroxidase involved in iron binding to transferrins in Dunaliella salina plasma membranes. The Journal of biological chemistry 282, 8658-8666.
Pober, J. S., Cotran, R. S. 1990. The role of endothelial cells in inflammation. Transplantation 50, 537-544.
Ponka, P., Lok, C.N., 1999. The transferrin receptor: role in health and disease. The international journal of biochemistry & cell biology 31, 1111-1137.
Poon, I.K.H., Lucas, C.D., Rossi, A.G., Ravichandran, K.S., 2014. Apoptotic cell clearance: basic biology and therapeutic potential. Nature Reviews Immunology 14, 166-180.

Poynter, S.J., DeWitte-Orr S.J., 2015. Length-dependent innate antiviral effects of double-stranded RNA in the rainbow trout (Oncorhynchus mykiss) cell line, RTG-2. Fish and Shellfish Immunology 46, 557-565.
Qian, Z. M., Li, H., Sun H., Ho, K. 2002. Targeted drug delivery via the transferrin receptor-mediated endocytosis pathway. Pharmacological reviews 54, 561-587.
Regoeczi, E., Hatton, M., 1980. Transferrin catabolism in mammalian species of different body sizes. American Journal of Physiology-Regulatory, Integrative and Comparative Physiology 238, R306-R310.
Reibman, J., Meixler, S., Lee, T.C., Gold, L.I., Cronstein, B.N., Haines, K.A., Kolasinski, S.L., Weissmann, G., 1991. Transforming growth factor beta 1, a potent chemoattractant for human neutrophils, bypasses classic signal-transduction pathways. Proceedings of the National Academy of Sciences 88, 6805-6809.
Ricciotti, E., FitzGerald, G.A., 2011. Prostaglandins and inflammation. Arteriosclerosis, thrombosis, and vascular biology 31, 986-1000.
Rieger, A.M., Havixbeck, J.J., Belosevic, M., Barreda, D.R., 2015. Teleost soluble CSF-1R modulates cytokine profiles at an inflammatory site, and inhibits neutrophil chemotaxis, phagocytosis, and bacterial killing. Developmental & Comparative Immunology 49, 259-266.
Rieger, A.M., Konowalchuk, J.D., Grayfer, L., Katzenback, B.A., Havixbeck, J.J., Kiemele, M.D., Belosevic, M., Barreda, D.R., 2012. Fish and Mammalian Phagocytes Differentially Regulate Pro-Inflammatory and Homeostatic Responses *In Vivo*. PLoS ONE 7, e47070.
Rodríguez, I., Novoa, B., Figueras, A., 2008. Immune response of zebrafish (Danio rerio) against a newly isolated bacterial pathogen Aeromonas hydrophila. Fish & shellfish immunology 25, 239-249.
Rossi, M.C., Zetter, B.R., 1992. Selective stimulation of prostatic carcinoma cell proliferation by transferrin. Proceedings of the National Academy of Sciences 89, 6197-6201.
Roush, E.D., Fierke, C.A., 1992. Purification and characterization of a carbonic anhydrase II inhibitor from porcine plasma. Biochemistry 31, 12536-12542.
Ruegemer, J.J., Ho, S.N., Augustine, J.A., Schlager, J.W., Bell, M., McKean, D., Abraham, R., 1990. Regulatory effects of transforming growth factor-beta on IL-2-and IL-4-dependent T cell-cycle progression. The Journal of Immunology 144, 1767-1776.
Ruszczyk, A., Forlenza, M., Joerink, M., Ribeiro, C.M.S., Jurecka, P., Wiegertjes, G.F., 2008. Trypanoplasma borreli cysteine proteinase activities support a conservation of function with respect to digestion of host proteins in common carp. Developmental & Comparative Immunology 32, 1348-1361.
Saha, R., Saha, N., Donofrio, R.S., Bestervelt, L.L., 2013. Microbial siderophores: a mini review. Journal of basic microbiology 53, 303-317.
Sakamoto, H., Sakamoto, N., Oryu, M., Kobayashi, T., Ogawa, Y., Ueno, M., Shinnou, M., 1997. A novel function of transferrin as a constituent of macromolecular activators of phagocytosis from platelets and their precursors. Biochemical and biophysical research communications 230, 270-274.
Sawyer, S. T., Krantz, S. B. 1986. Transferrin receptor number, synthesis, and endocytosis during erythropoietin-induced maturation of friend virus-ifected erythroid cells. Journal of Biological Chemistry 261, 9187-9195.

Schade, A.L., Caroline, L., 1944. RAW HEN EGG WHITE AND THE ROLE OF IRON IN GROWTH INHIBITION OF SHIGELLA DYSENTERIAE, STAPHYLOCOCCUS AUREUS, ESCHERICHIA COLI AND SACCHAROMYCES CEREVISIAE. Science (New York, N.Y.) 100, 14-15.
Schaeffer, E., Lucero, M.A., Jeltsch, J.M., Py, M.C., Levin, M.J., Chambon, P., Cohen, G.N., Zakin, M.M., 1987. Complete structure of the human transferrin gene. Comparison with analogous chicken gene and human pseudogene. Gene 56, 109-116.
Schaible, U.E., Kaufmann, S.H.E., 2004. Iron and microbial infection. Nat Rev Micro 2, 946-953.
Schell, D., Borowy, N.K., Overath, P., 1991. Transferrin is a growth factor for the bloodstream form ofTrypanosoma brucei. Parasitology research 77, 558-560.
Schneider, C., Owen, M. J., Banville, D., Williams, J. G. 1984. Primary structure of human transferrin receptor deduced fro mthe mRNA sequence. Nature 311, 675-678.
Sekyere, E.O., Dunn, L.L., Suryo Rahmanto, Y., Richardson, D.R., 2006. Role of melanotransferrin in iron metabolism: studies using targeted gene disruption in vivo. Blood 107, 2599-2601.
Serhan, C.N., Savill, J., 2005. Resolution of inflammation: the beginning programs the end. Nature immunology 6, 1191-1197.
Siepl, C., Bodmer, S., Frel, K., MacDonald, H.R., Martin, R.D., Hofer, E., Fontana, A., 1988. The glioblastoma- derived t cell suppressor factor/transforming growth factor- β2 inhibits t cell growth without affecting the interaction of interleukin 2 with its receptor. European journal of immunology 18, 593-600.
Siewe, L., Bollati–Fogolin, M., Wickenhauser, C., Krieg, T., Müller, W., Roers, A., 2006. Interleukin- 10 derived from macrophages and/or neutrophils regulates the inflammatory response to LPS but not the response to CpG DNA. European journal of immunology 36, 3248-3255.
Skaar, E.P., 2010. The battle for iron between bacterial pathogens and their vertebrate hosts. PLoS pathogens 6.
Soehnlein, O., Lindbom, L., 2010. Phagocyte partnership during the onset and resolution of inflammation. Nature Reviews Immunology 10, 427-439.
Spittler, A., Schiller, C., Willheim, M., Tempfer, C., Winkler, S., Boltz-Nitulescu, G., 1995. IL-10 augments CD23 expression on U937 cells and down-regulates IL-4-driven CD23 expression on cultured human blood monocytes: effects of IL-10 and other cytokines on cell phenotype and phagocytosis. Immunology 85, 311.
Sriyosachati, S., Cox, C.D., 1986. Siderophore-mediated iron acquisition from transferrin by Pseudomonas aeruginosa. Infection and Immunity 52, 885-891.
Stafford, J.L., 2003. Analysis of the mechanisms of macrophage activation in fish. University of Alberta.
Stafford, J.L., Belosevic, M., 2003. Transferrin and the innate immune response of fish: identification of a novel mechanism of macrophage activation. Developmental & Comparative Immunology 27, 539-554.
Stafford, J.L., Neumann, N.F., Belosevic, M., 2001. Products of proteolytic cleavage of transferrin induce nitric oxide response of goldfish macrophages. Developmental & Comparative Immunology 25, 101-115.

Stafford, J.L., Wilson, E.C., Belosevic, M., 2004. Recombinant transferrin induces nitric oxide response in goldfish and murine macrophages. Fish & shellfish immunology 17, 171-185.
Steverding, D., 2000. The transferrin receptor of Trypanosoma brucei. Parasitology international 48, 191-198.
Takemura, R., Werb, Z., 1984. Secretory products of macrophages and their physiological functions. The American Journal of Physiology 246, C1-9.
Takenaka, K., Suzuki, S., Sakai, N., Kassell, N.F., Yamada, H., 1995. Transferrin induces nitric oxide synthase mRNA in rat cultured aortic smooth muscle cells. Biochemical and biophysical research communications 213, 608-615.
Takenaka, K.V., Sakai, N., Murase, S., Kuroda, T., Okumura, A., Sawada, M., 2000. Elevated transferrin concentration in cerebral spinal fluid after subarachnoid hemorrhage. Neurological research 22, 797-801.
Tang, S., Leung, J.C., Tsang, A.W., Lan, H.Y., Chan, T.M., Lai, K.N., 2002. Transferrin up-regulates chemokine synthesis by human proximal tubular epithelial cells: implication on mechanism of tubuloglomerular communication in glomerulopathic proteinura. Kidney international 61, 1655-1665.
Thompson, G.J., Crozier, Y.C., Crozier, R.H., 2003. Isolation and characterization of a termite transferrin gene up-regulated on infection. Insect molecular biology 12, 1-7.
Underhill, D.M., Goodridge, H.S., 2012. Information processing during phagocytosis. Nature Reviews Immunology 12, 492-502.
Unuma, T., Nakamura, A., Yamano, K., Yokota, Y., 2010. The sea urchin major yolk protein is synthesized mainly in the gut inner epithelium and the gonadal nutritive phagocytes before and during gametogenesis. Molecular reproduction and development 77, 59-68.
Unuma, T., Yamamoto, T., Akiyama, T., Shiraishi, M., Ohta, H., 2003. Quantitative changes in yolk protein and other components in the ovary and testis of the sea urchin Pseudocentrotus depressus. The Journal of experimental biology 206, 365-372.
Uribe, C., Folch, H., Enriquez, R., Moran, G., 2011. Innate and adaptive immunity in teleost fish: a review. Veterinarni Medicina 56, 486-503.
van der Kraan, M.I., Groenink, J., Nazmi, K., Veerman, E.C., Bolscher, J.G., Amerongen, A.V.N., 2004. Lactoferrampin: a novel antimicrobial peptide in the N1-domain of bovine lactoferrin. Peptides 25, 177-183.
Vostrejs, M., Moran, P.L., Seligman, P.A., 1988. Transferrin synthesis by small cell lung cancer cells acts as an autocrine regulator of cellular proliferation. Journal of Clinical Investigation 82, 331-339.
Voyiatzaki, C., Soteriadou, K., 1992. Identification and isolation of the Leishmania transferrin receptor. Journal of Biological Chemistry 267, 9112-9117.
Wahl, S.M., Hunt, D.A., Wakefield, L.M., McCartney-Francis, N., Wahl, L.M., Roberts, A.B., Sporn, M.B., 1987. Transforming growth factor type beta induces monocyte chemotaxis and growth factor production. Proceedings of the National Academy of Sciences 84, 5788-5792.
Wahl, S.M., Hunt, D.A., Wong, H.L., Dougherty, S., McCartney-Francis, N., Wahl, L.M., Ellingsworth, L., Schmidt, J., Hall, G., Roberts, A., 1988. Transforming growth factor-beta is a potent immunosuppressive agent that inhibits IL-1-dependent lymphocyte proliferation. The Journal of Immunology 140, 3026-3032.

Wallace, J.L., Keenan, C.M., Granger, D.N., 1990. Gastric ulceration induced by nonsteroidal anti-inflammatory drugs is a neutrophil-dependent process. American Journal of Physiology-Gastrointestinal and Liver Physiology 259, G462-G467.
Wang, J., Pantopoulos, K., 2011. Regulation of cellular iron metabolism. Biochemical Journal 434, 365-381.
Ward, P.P., Conneely, O.M., 2004. Lactoferrin: Role in iron homeostasis and host defense against microbial infection. Biometals 17, 203-208.
Ward, P.P., Mendoza-Meneses, M., Cunningham, G.A., Conneely, O.M., 2003. Iron status in mice carrying a targeted disruption of lactoferrin. Molecular and cellular biology 23, 178-185.
Weinberg, E.D., 2009. Iron availability and infection. Biochimica et Biophysica Acta (BBA)-General Subjects 1790, 600-605.
Wiseman, D.M., Polverini, P.J., Kamp, D.W., Leibovich, S.J., 1988. Transforming growth factor-beta (TGFβ) is chemotactic for human monocytes and induces their expression of angiogenic activity. Biochemical and biophysical research communications 157, 793-800.
Wong, J.M., Billiar, T.R., 1994. Regulation and function of inducible nitric oxide synthase during sepsis and acute inflammation. Advances in pharmacology (San Diego, Calif.) 34, 155-170.
Wuebbens, M.W., Roush, E.D., Decastro, C.M., Fierke, C.A., 1997. Cloning, sequencing, and recombinant expression of the porcine inhibitor of carbonic anhydrase: a novel member of the transferrin family. Biochemistry 36, 4327-4336.
Xie, H., Huff, G., Huff, W., Balog, J., Holt, P., Rath, N., 2002a. Identification of ovotransferrin as an acute phase protein in chickens. Poultry science 81, 112-120.
Xie, H., Huff, G.R., Huff, W.E., Balog, J.M., Rath, N.C., 2002b. Effects of ovotransferrin on chicken macrophages and heterophil-granulocytes. Developmental & Comparative Immunology 26, 805-815.
Xie, H., Newberry, L., Clark, F., Huff, W., Huff, G., Balog, J., Rath, N., 2002c. Changes in serum ovotransferrin levels in chickens with experimentally induced inflammation and diseases. Avian diseases 46, 122-131.
Xie, H., Rath, N., Huff, G., Balog, J., Huff, W., 2001. Inflammation-induced changes in serum modulate chicken macrophage function. Veterinary immunology and immunopathology 80, 225-235.
Yang, D., Biragyn, A., Kwak, L.W., Oppenheim, J.J., 2002. Mammalian defensins in immunity: more than just microbicidal. Trends in immunology 23, 291-296.
Yang, L., Zhou, L., Gui, J.F., 2004. Molecular basis of transferrin polymorphism in goldfish (Carassius auratus). Genetica 121, 303-313.
Yipp, B.G., Petri, B., Salina, D., Jenne, C.N., Scott, B.N.V., Zbytnuik, L.D., Pittman, K., Asaduzzaman, M., Wu, K., Meijndert, H.C., 2012. Infection-induced NETosis is a dynamic process involving neutrophil multitasking in vivo. Nature medicine 18, 1386-1393.
Yoshiga, T., Georgieva, T., Dunkov, B.C., Harizanova, N., Ralchev, K., Law, J.H., 1999. Drosophila melanogaster transferrin. European journal of biochemistry 260, 414-420.
Yoshiga, T., Hernandez, V.P., Fallon, A.M., Law, J.H., 1997. Mosquito transferrin, an acute-phase protein that is up-regulated upon infection. Proceedings of the National Academy of Sciences of the United States of America 94, 12337-12342.

Yoshikawa, T., Naito, Y., 2000. The role of neutrophils and inflammation in gastric mucosal injury. Free radical research 33, 785-794.
Young, H.A., 1996. Regulation of interferon-γ gene expression. Journal of interferon & cytokine research 16, 563-568.
Yun, E.Y., Lee, J.K., Kwon, O.Y., Hwang, J.S., Kim, I., Kang, S.W., Lee, W.J., Ding, J.L., You, K.H., Goo, T.W., 2009. Bombyx mori transferrin: genomic structure, expression and antimicrobial activity of recombinant protein. Developmental and comparative immunology 33, 1064-1069.
Zakin, M.M., 1992. REGULATION OF TRANSFERRIN GENE-EXPRESSION. Faseb J. 6, 3253-3258.
Zhang, X., Mosser, D.M., 2008. Macrophage activation by endogenous danger signals. The Journal of pathology 214, 161-178.