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Contributions of transferrin to acute inflammation in the goldfish, *C. auratus*

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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, by depriving iron from invading pathogens. Recently cleaved transferrin products was shown to activate macrophages *in vitro*. We now use an *in vivo* model of self-resolving peritonitis in goldfish, coupled with gene expression and protein analysis to evaluate the contributions of cleaved transferrin to acute inflammation. We show, for the first time, that cleaved transferrin products are produced *in vivo* early during an acute inflammatory response. These cleaved transferrin fragments were produced during pathogen-induced, but not sterile, inflammation. Both macrophages and neutrophils were able to contribute to transferrin cleavage. However, only macrophages contributed to this innate process through inducible expression of transferrin. The appearance of transferrin cleavage products *in vivo* correlated with the influx of leukocytes but did not necessarily correlate the induction of robust respiratory burst and nitric oxide responses. Overall, this study adds to a growing body of 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.

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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

Abbreviations: cDNA, complementary DNA; DAMP, danger associated molecular pattern; PAMP, pathogen associated molecular pattern; NET, neutrophil extracellular trap; NO, nitric oxide; poly(I:C), Polyinosinic-Polycytidylic acid; RNA, ribonucleic acid; ROS, reactive oxygen species.

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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 enzymes primarily responsible for facilitating transferrin cleavage have 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).

Our goal for this study was to assess the appearance *in vivo* of transferrin cleavage products during the teleost acute inflammatory process. We hypothesized that cleaved transferrin products would be produced locally within an immune challenge site, contribute to antimicrobial responses of infiltrating leukocytes and display differential kinetics depending on the inflammatory stimulus. To test this hypothesis, we 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, we show endogenous transferrin cleavage products at the inflammatory site, which are not seen in the serum. Finally, we show that these resultant peptides are selectively produced during pathogen induced, but not sterile, inflammation.

2. Materials and methods

2.1. Animals

Goldfish (*C. auratus* L.) 7–11 cm in length were purchased from Mount Parnell (Mercersburg, PA) and maintained in the Aquatic Facility of the Department of Biological Sciences, University of Alberta. The fish were held at 20 °C in a flow-through water system on a simulated natural photoperiod. Animals were maintained according to the guidelines of the Canadian Council of Animal Care. Protocols were approved by the University of Alberta Animal Care and Use Committee (ACUC-Biosciences; Protocol Number 706).

2.2. Preparation of pathogens and pathogen mimics

Zymosan (Alfa Aesar) was suspended at a concentration of 50 mg/mL in 1× PBS$^{-/-}$ (no calcium/no magnesium) and stored at 4 °C until use in experiments. Pathogenic *Aeromonas veronii* was isolated from an active *C. auratus* infection at the University of Alberta. *A. veronii* were grown at room temperature in trypticase-soy-agar (TSA) broth (BD) overnight. The number of colony forming units (CFU)/mL was determined by cultivation on TSA agar plates. Bacteria were heat killed by incubation of enumerated cultures in an 80 °C water bath for 1 h with gentle agitation every 15 min. Polyinosinic-Polycytidylic acid, poly(I:C), (Sigma) was suspended at a concentration of 50 mg/mL in sterile 1× PBS$^{-/-}$ and stored at − 20 °C until use.

2.3. Transferrin gene expression

Goldfish were injected intraperitoneally with 2.5 mg zymosan, 5.0 × 10^{6} C.F.U of heat-killed *A. veronii*, 200 μg of Poly(I:C), or sterile 1× PBS$^{-/-}$. Samples were injected in 100 μL 1× PBS$^{-/-}$. Goldfish peritoneal leukocytes were used for the study of transferrin gene expression. Total peritoneal leukocytes were isolated by peritoneal lavage with ice-cold 1× PBS$^{-/-}$. Leukocytes were separated in subpopulations as previously described (Havixbeck et al., 2016; Rieger et al., 2015) with minor modifications. Briefly, the cell suspension was layered on 51% Percoll (GE Healthcare) and centrifuged at 400×*g* for 25 min. Macrophages, monocytes, and lymphocytes located at the medium-Percoll interface were collected, washed with 1× PBS$^{-/-}$, and centrifuged at 311×*g* for 10 min. Primary macrophages and monocytes were separated from lymphocytes based on their differential adherence to plastic surfaces (Clem et al., 1984; Neumann et al., 2000). In short, leukocytes from the medium-Percoll interface and allowed to adhere to plastic (Corning) in incomplete MGFL-15 medium for 4 h at 20 °C. Media was prepared as previously described by Neumann et al. (1998). Non-adherent lymphocytes were separated from primary macrophages and monocytes by decanting the supernatants. Primary macrophages and monocytes were flash frozen in liquid nitrogen and subsequently used for RNA extraction.

Isolation of primary neutrophils was performed as previously described (Havixbeck et al., 2016). In short, the neutrophil-erythrocyte pellet was washed with 1× PBS$^{-/-}$, suspended in ACK lysing buffer (Gibco) for 3 min, and subsequently washed twice with 1× PBS$^{-/-}$ at 311 × g for 10 min. The remaining neutrophils were then used for RNA extraction. Isolated cellular preparations were stained using a HEMA 3 stain set (Fisher Scientific) according to the manufacturer's specifications.

Total RNA was extracted from isolated macrophages and neutrophils using an RNA extraction kit for cells (Qiagen), according to manufacturer's instructions. Total RNA was used for cDNA synthesis using SMARTScribe Reverse Transcriptase (Clontech) according to manufactures protocol. cDNA was stored at −20 °C prior to use in PCR reactions.

RT-PCR reactions used to amplify transferrin, and β-actin PCR reactions were performed using primer pairs ordered from Integrated DNA Technologies (Table 1). The primer pairs were designed to amplify PCR products of 1430 and 350 b.p. for transferrin and β-actin, respectively. Semi-quantitative RT-PCR amplification was performed by combining the following reagents into a master mix such that each reaction contained the following: 40.3 μL sterile H_2O, 5 μL 10× PCR buffer (with 1.5 mM $MgCl_2$), 0.8 μL dNTPs (25 mM), 1.2 μL of forward and reverse primers (25 mM), and 0.5 μL DNA polymerase (5 U/μL). The master mix was gently mixed and

Table 1
Primer sequences used for RT-PCR.

Primer	Sequence (5′–3′)
Transferrin forward	GCT CAT CTC GTT TCT GGC GTG CC
Tansferrin reverse	GAT CAG CAC CAG GAG CGC AGC C
β-actin forward	CGA GCT GCG TGT TGC CCC TGA G
β-actin reverse	CGG CCG TGG TGG TGA AGC TGT AG

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