Identification and Molecular Characterization of Zebrafish (*Danio rerio*) Leukocyte Immune-Type Receptors (DrLITRs)

by

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ABSTRACT

In response to foreign invaders, innate immune cells use cell surface-expressed receptors to trigger intracellular signalling cascades that control various effector responses designed to destroy microbes. These specialized receptors are known as immunoregulatory receptors and structurally they exist as stimulatory and inhibitory types. In channel catfish (*Ictalurus punctatus*), the leukocyte immune-type receptor (IpLITR) family consists of multiple members, each with variable signalling abilities. Using *in vitro* approaches, our previous work has shown that IpLITRs also function as potent regulators of antimicrobial responses including phagocytosis, degranulation, cytokine secretion and cytotoxicity. To better understand the potential roles of LITRs *in vivo*, my thesis research focused on using zebrafish as a model organism to further examine teleost LITRs. I cloned and sequenced four putative *Danio rerio* (Dr)LITRs, termed DrLITR 1.1, DrLITR 1.2, DrLITR 15.1 and DrLITR 23.1 that were identified in the zebrafish genome. These receptors are related to IpLITRs and they are also distantly related to important immunoregulatory receptors found in mammals (e.g.FcRLs, SIGLECs, and CEACAMs). Structurally, DrLITRs 1.1 and 1.2 contain three extracellular immunoglobulin (Ig) domains, a transmembrane (TM) region and a cytoplasmic tail (CYT) containing both immunoreceptor tyrosine-based activation motif (ITAM) and immunoreceptor tyrosine-based inhibition motif (ITIM). DrLITR 15.1 contains four Ig domains, a TM segment and a CYT region with two ITIMs and one immunoreceptor tyrosine-based switch motif (ITSM). DrLITR 23.1 contains six Ig domains, a positively-charged TM segment and a CYT region devoid of any recognizable tyrosine-based motifs. I also examined the expression of DrLITRs throughout ontogeny and adulthood with and without an immunostimulant. These results showed that all four DrLITR-

types are expressed as early as 1 hour post fertilization (hpf) and they remain present throughout embryonic development and adulthood. Although the pro-inflammatory cytokine IL1β displayed significant increases in expression at 3, 6, 8 and 12 hours using a visceral cavity based inflammation assay, the expression profiles of DrLITR 1.1, DrLITR 1.2, DrLITR 15.1 and DrLITR 23.1 displayed different trends over the same period following an intraperitoneal injection of zebrafish with 1 μg/mL zymosan. DrLITR 1.1 was significantly upregulated at 12 hours after zymosan exposure while DrLITR 1.2 was significantly upregulated at 3, 6 and 12 hours post zymosan injection. DrLITR 15.1 displayed no statistically significant upregulation/downregulation patterns at any time point while DrLITR 23.1 was downregulated at 24 hours after zymosan injection. Overall, this work sets the stage for establishing zebrafish as a model system to study these novel immunoregulatory receptors during various developmental stages of fish and during pathogen-induced inflammatory responses.

PREFACE

This thesis is the original work of Hima Varsha Gurupalli. Zebrafish were used during the completion of this research and approval was obtained from the Animal Care and Use Committee at the University of Alberta (AUP #00000816).

No part of this thesis has been previously published.

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LIST OF ABBREVIATIONS

- aa — Amino acids
- AD293 — Semi-adherent version of HEK293 cells
- ADCC — Antibody-dependent cellular cytotoxicity
- Akt — Protein kinase B
- BLAST — Basic Local Alignment Search Tool
- BLASTp — Protein BLAST
- bp — Base pair
- C3-C9 — Complement components 3-9
- CD — Cluster of differentiation
- CD3ζ-L — CD3 zeta-like subunit
- CD94/NKG2 — Family of C-type lectin receptors on NK cells
- cdc42 — Cell division cycle 42
- cDNA — Complementary deoxyribonucleic acid
- CEACAM — Carcinoembryonic antigen-related cell adhesion molecule
- CHIR — Chicken Ig-like receptor
- Con A — Concanavalin A
- Csk — C-terminal Src kinase
- C_t — Threshold cycle
- CTL — Cytotoxic T lymphocyte
- CYT — Cytoplasmic tail
- D1-D6 — Immunoglobulin domains 1-6
- DAP — DNAX-activation protein
- DNA — Deoxyribonucleic acid
- dpf — Days post fertilization
- DrLITR — *Danio rerio* leukocyte immune-type receptor
- EF1α — Elongation Factor 1 alpha

- ERK Extracellular signal-regulated kinase
- EST Expressed sequence tag
- Fc Fragment crystallizable
- FcR Fragment crystallizable receptor
- FcRL Fragment crystallizable receptor-like
- FcRγ FcR γ-chain
- FcRγL FcR γ-chain-like
- Fcα/μR Fc receptor for both IgA and IgM
- FcαR Fc receptor for IgA
- Fcγ Fc portion of Igγ
- Fcε Fc portion of Igε
- FcεR Fc receptor for IgE
- FcεRI High-affinity Fc receptor for IgE
- FcμR Fc receptor for IgM
- FLAG Epitope tag encoding amino acid residues DYKDDDDK
- Fwd forward
- GEF Guanine exchange factor
- Grb Growth factor receptor-bound
- GST Glutathione S-transferase
- GTPase Guanosine triphosphatase
- HA Hemagglutinin
- HEK Human embryonic kidney
- hpf Hours post fertilization
- Ig Immunoglobulin
- IgA Immunoglobulin A
- IgD Immunoglobulin D
- IgG Immunoglobulin G
- IgM Immunoglobulin M

- IgSF Immunoglobulin superfamily
- IL Interleukin
- IL-1β Interleukin 1 beta
- IpFcRI Channel catfish Fc receptor I
- IpLITR *Ictalurus punctatus* leukocyte immune-type receptor
- ITAM Immunoreceptor tyrosine-based activating motif
- ITIM Immunoreceptor tyrosine-based inhibitory motif
- ITSM Immunoreceptor tyrosine-based switch motif
- kb kilobases
- KIR Killer cell Ig-like receptor
- LB Luria-Bertani
- LILR Leukocyte Ig-like receptor
- LITR Leukocyte immune-type receptor
- LMIR Leukocyte mono-immunoglobulin-like receptor
- LPS Lipopolysaccharide
- LRC Leukocyte receptor complex
- Lyn Lck/Yes-related novel protein tyrosine kinase
- mAb Monoclonal antibody
- MAC Membrane attack complex
- MBL Mannose-binding lectins
- MBSU Molecular Biology Service unit
- MEK Mitogen-activated protein kinase
- MHC Major histocompatibility class
- miRNA microRNA
- MLC Mixed leukocyte culture
- MPO Myeloperoxidase
- mRNA Messenger ribonucleic acid
- MS-222 Tricaine mesylate

- Nck Non-catalytic region of tyrosine kinase adaptor protein
- NITR Novel immune-type receptor
- NK Natural killer
- NO Nitric oxide
- PAMP Pathogen-associated molecular pattern
- PBL Peripheral blood leukocyte
- PBS Phosphate buffered saline
- PCR Polymerase chain reaction
- PDK1 Phosphoinositide-dependent kinase 1
- PECAM Platelet endothelial cell adhesion molecule
- PI3K Phosphoinositide 3-kinase
- PIR Paired Ig-like receptor
- PKC Protein kinase C
- PMA Phorbol 12-myristate 13-acetate
- PRR Pattern recognition receptor
- PSG Pregnancy specific glycoprotein.
- PSI-BLAST Position Specific iterative (PSI) BLAST
- pSyk Phosphorylated Syk
- qPCR Quantitative polymerase chain reaction
- Rac Ras-related C3 botulinum toxin substrate
- Ras Rat sarcoma 2 viral oncogene homolog
- RBL Rat basophilic leukemia
- Rho Rat sarcoma viral oncogene homolog
- RNA Ribonucleic acid
- RT-PCR Reverse transcription polymerase chain reaction
- rtIL-1β Recombinant form of IL1β
- Rvrs Reverse
- SFK Src family kinase

- SH2 Src homology 2
- SH2D1A SH2-containing adaptor protein 1A
- SHIP SH2 domain-containing inositol 5’-phosphatase
- SHP SH2 domain-containing phosphatase
- SIGLEC Sialic acid-binding Ig-type lectin
- SMART Simple Modular Architecture Research Tool
- Src Sarcoma viral oncogene homolog
- Syk spleen tyrosine kinase
- TAE Tris base, acetic acid, Ethylenediaminetetraacetic acid
- TL Tupfel long fin
- TLR Toll-like receptor
- TM Transmembrane
- TNF Tumor necrosis factor
- TNFR TNF receptor
- TNP Trinitrol-phenol
- UTR Untranslated region
- VCAM Vascular cell adhesion protein
- WAVE Wiskott Aldrich syndrome protein family verprolin-homologous protein
- XMIV *Xenopus* MHC-linked Ig superfamily V genes
- ZFL Zebrafish liver cell line
- αHA Anti-hemagglutinin tag

CHAPTER I

INTRODUCTION

1.1 INTRODUCTION

The immune system is vital for host protection from infectious bacteria and viruses. Innate immunity encompasses several non-specific responses that are activated immediately upon microbe encounter. One component of innate immunity offers protection via physical barriers such as skin and mucus. Beyond these barriers, innate immune responses rely on the actions of specific cellular subsets. Certain cell subsets sense and respond to external environmental cues via signal transduction through cell surface-expressed receptors that trigger intracellular signalling cascades to generate specific cellular responses. To balance cellular activation and inhibition, immunoregulatory receptors include both stimulatory and inhibitory types that are largely defined by the motifs required for signalling. These receptors bind a wide array of ligands, ranging from damaged/injured host cell markers to pathogen-derived markers, and translate this information to immune cells via specific intracellular signalling pathways. Immunoregulatory receptors that belong to immunoglobulin superfamily (IgSF), meaning they contain one or more extracellular Ig-like domains, have been implicated in mediating a wide array of cell effector responses throughout vertebrates..

Many IgSF members have been identified in non-mammalian organisms, such as birds, amphibians and fish, but they are only well-characterized in mammals. Innate immunity consists of many known conserved evolutionary processes, such as phagocytosis (engulfment of large particles) and degranulation (release of anti-microbial components via granules) . Therefore, studying non-mammalian models can expand our understanding of various innate

immune responses. A teleost immunoregulatory receptor family known as leukocyte immune-type receptors (LITRs) were discovered in channel catfish over a decade ago (Stafford *et al.*, 2006). As with many receptors of the IgSF family, LITRs contain both stimulatory and inhibitory receptor-types. These receptors have the potential to regulate a wide array of cellular responses, such as degranulation and phagocytosis, when expressed in mammalian cell lines (Cortes *et al.*, 2012, 2014). Additionally, an inhibitory IpLITR-type mediates both stimulatory and inhibitory responses using novel mechanisms, which showcases its signalling versatility (Montgomery *et al.*, 2012; Cortes *et al.*, 2014; Fei *et al.*, 2016; Zwozdesky *et al.*, 2017). These novel characteristics of LITRs highlight their need to be studied and characterized as they may broaden our understanding of the signalling potentials of innate immune responses. As all functional work with LITRs has been performed *in vitro*, the functional significance of these receptors *in vivo* is still yet to be examined.

1.2 THESIS OBJECTIVES

The biggest challenge to LITR research is that all work has been limited to *in vitro* studies. Therefore, the main goal of my thesis was to identify zebrafish LITRs and establish zebrafish as an ontogeny and inflammation model for examining the expression patterns of LITRs *in vivo*. The specific aims of my thesis were:

1. Discovery of candidate LITR-types in the zebrafish genome
2. Amplify, clone, and sequence putative zebrafish LITR-types
3. Examination of LITR expression during zebrafish development
4. Examination of LITR expression in adult fish using a visceral cavity based inflammation model

1.3 THESIS OVERVIEW

The second chapter of this thesis will highlight the literature surrounding mammalian and fish immunoregulatory receptors. Specifically, I will talk about the LITR research that has been performed to date and why these receptors are an excellent model system to study the signalling of innate immune processes. Chapter III details all the materials and methods that were used to complete this thesis. In Chapter IV, I use bioinformatics approaches, such as BLAST and EST databases, to identify candidate LITR-types in zebrafish. Of the resulting top hits, I pursued four LITR-types based on the presence of various tyrosine-based motifs in their cytoplasmic tails. Consistent with the previous naming conventions of IpLITRs, I named zebrafish (*Danio rerio*) LITRs as DrLITRs. I named the four DrLITRs based on their chromosomal location: DrLITR 1.1, DrLITR 1.2, DrLITR 15.1 and DrLITR 23.1. Furthermore, *in silico* analysis has revealed the fact that LITRs encode extracellular domains pertaining to various mammalian receptors into one receptor suggesting that LITRs and mammalian innate immune receptors were once encoded on the same chromosome. In chapter V, I developed a qPCR assay to measure the expression of LITRs during ontogeny and adulthood. I found that DrLITRs are ubiquitously expressed regardless of the age of the fish. During ontogeny, all four LITRs showed variable expression patterns suggesting that they may have different developmental roles. Additionally, after exposure to zymosan, all four DrLITRs expressed in the visceral cavity of the fish displayed variable upregulation/downregulation patterns suggesting that they may have different functional roles during an immune response. In chapter VI, I will provide a summary of my results in relation to both data chapters and provide some future directions of LITR research. Chapter VII contains all the references used in this thesis.

CHAPTER II

LITERATURE REVIEW

2.1 GENERAL OVERVIEW OF INNATE IMMUNITY

The immune system is divided into two main branches: innate and adaptive. All eukaryotic organisms contain an innate immune system. It acts as the first line of defense during immune challenges. This includes anatomical and chemical barriers such as skin and mucus. If these barriers are breached, then the cellular components of the innate immune system are activated. This primarily includes specific cell sub-types and their mediation of functional responses during pathogen challenge. Innate immune cells are very well characterized in mammalian systems and have been found to be evolutionarily conserved throughout vertebrates. These include macrophages, natural killer (NK) cells, dendritic cells, and neutrophils. These immune cells are capable of mediating various antimicrobial functions such as phagocytosis, inflammation and degranulation in response to various microbes (e.g. bacteria, viruses, and parasites). Additionally, these immune cells are heavily involved in maintaining homeostasis, recognizing stressed host cells and repairing damaged tissues.

The adaptive immune system is immunity acquired over a period of time depending on the types of pathogens a host has encountered (Yatim and Lakkis, 2015). Additionally, the adaptive system requires the host to encounter a pathogen before the immune system can act and this takes a long time to accumulate. For example, when a pathogen first infiltrates the host body, the highly specialized cells of the adaptive immune system (T and B cells) are slow to provide defense as they are learning the molecular signature of the intruder. This results in the creation of memory B and T cells (Bonilla and Oettgen, 2010). Any subsequent encounter with

the same pathogen results in a robust immune response, which rapidly clears out the infection. The memory B and T cells are only specific to the pathogens they encounter. If a new pathogen is encountered, then the whole process is repeated again. In general, B cells create specific antibodies, also known as immunoglobulins, in response to pathogen encounters. Memory B cells express these antibodies on their surfaces and, in the case of re-exposure to the same pathogen, these memory B-cells are quickly recruited to deal with the pathogenic challenge. Antibodies have a distinctive "Y" shape. The tips of the "Y" recognize a particular molecular signature that belong to a specific pathogen. In other words, each antibody recognizes one specific component of one pathogen. In addition, antibodies contain a fragment crystallizable (Fc) region that communicates the pathogen information to receptors which, in turn, serve to mediate various immune responses. All jawed vertebrates have a classical adaptive immunity featuring B cells, T cells, and antibodies but jawless vertebrates such as lamprey and hagfish possess an alternate version of the adaptive system. Jawless vertebrates do not contain the same cell types as the classical adaptive system described in mammals (reviewed by Herrin and Cooper, 2010). This is due to the fact that jawless vertebrates do not possess immunoglobulins, the extracellular portion of mammalian adaptive immune receptors. However, they do possess leucine-rich repeats in the extracellular portion of their receptors that function similarly to mammalian receptors. Therefore, they are considered to have an alternate version of the adaptive system. In other words, they have mammalian "adaptive immune cell-like" lineages but do not truly have the same immune cells.

Adaptive immunity is long-lasting due to memory cells as well as a highly specific response catered to specific types of pathogens. Conversely, the innate immune system is not

as specific. It can differentiate between different classes of pathogens (i.e. fungus vs. bacteria vs. viruses) but it cannot specifically differentiate between specific types of fungi or viruses (Akira, Uematsu and Takeuchi, 2006). Innate immunity recognizes highly conserved features that a family of pathogens share called pathogen associated-molecular patterns (PAMPs) (reviewed by Kumagai and Akira, 2010). These are recognized by pattern recognition receptors (PRRs). A well-described family of PRRs in mammals are called toll-like receptors (TLRs). There are 13 TLRs characterized in mammals, termed TLR1-TLR13, which are expressed by various immune cell-types. Each TLR recognizes specific types of PAMPs. For example, TLR4 recognizes lipopolysaccharides, a PAMP that is characteristic of gram-negative bacterial membranes and it initiates an immune response to clear the bacterial infection. Innate immunity is particularly useful for newborn organisms as the germline-encoded innate immune system is their main source of defense and it continues being the first line of defense for all stages of life. Due to the reasons mentioned above, innate immunity is considered non-specific relative to the highly specific nature of adaptive immunity.

In this review, I will talk about the innate immune system in mammals and teleost fishes to set the stage for my research. I will also talk about some key innate immune processes and their importance in host protection. I will also be focusing on immunoregulatory receptors as my research is focused on one such receptor family, known as Leukocyte Immune-Type receptors (LITRs). Initially, these receptors were discovered in the Channel catfish (*Ictalurus punctatus*) so I will talk about why this specific fish species is a good model to study innate immunity. Finally, I will provide an update on what we have learned about IpLITRs over the past decade.

2.1.1 Innate immunity: physical barriers

A major function of the immune system is to act as the first line of defense to protect host tissues from the infiltration of various microbes and pathogens (reviewed by Iwasaki and Medzhitov, 2015; Riera Romo, Pérez-Martínez and Castillo Ferrer, 2016). The skin is the first anatomical/physical barrier that a pathogen will encounter. All vertebrates contain the dermis and epidermis cellular layers that act to block pathogen entry. These layers contain many specialized immune cells that migrate continuously to respond to various pathogenic challenges.

In fish, the epidermal layer is protected by mineralized scales and actin-rich filaments (reviewed by Sire and Akimenko, 2004; Alibardi, 2006). As a result, it becomes extremely difficult for microbes to penetrate this rigid exterior as long as it remains intact. Additionally, highly specialized cells (i.e. goblet cells) secrete a viscous substance, known as mucus. This is effective for trapping pathogens in one area but also has many other preventative measures. Mucus generally contains antibody proteins, low pH, hydrolytic enzymes; all of which have antimicrobial properties. If pathogens are able to infiltrate this physical layer, then various immune cellular effector responses are induced in the infected tissue.

2.1.2 Innate immunity: cell effector responses

Immune cells are generally divided into two main cell lineages: myeloid and lymphoid. Myeloid cells include the majority of the innate immune cell-types including macrophages, granulocytes as well as dendritic cells (Rombout *et al.*, 2005). Lymphoid cells are usually destined to differentiate into adaptive immune cells, but NK cells are the exception. NK cells are

considered to have a major role in the innate immune system due to their ability to recognize stressed, infected, and/or transformed (i.e. neoplastic) host cells.

The cells of the myeloid lineage are the classical innate immune cells that mediate the majority of the primary innate immune processes during initial pathogen challenge. Some of these responses include degranulation, cytokine secretion, phagocytosis and activation of the complement cascade. These cell-types are involved in mediating a wide array of immune responses that are evolutionarily conserved across all vertebrates. During an infection, it is critical for immune cells to migrate to the site of pathogen entry where they then induce their effector antimicrobial responses as part of an overall process called inflammation.

Phagocytosis is defined as an actin-dependent engulfment process of targets greater than 0.5 μm in size. This is an ancient and evolutionary conserved process that is seen in both vertebrates and invertebrates. Degranulation is the process of releasing granules filled with antimicrobial components that are targeted towards pathogens. This process is performed mainly by granulocytes (neutrophils, basophils and eosinophils and mast cells). Granules contained the following enzymes: myeloperoxidase (MPO), proteinase-3, cathepsin G and elastase, and acidic hydrolases, as well as antimicrobial proteins such as cathlicidin and lactoferrin (Morel, Doussiere, & Vignais, 1991). In teleost fishes, it was observed that homologues of MPO, proteinase-3, cathepsin G and elastase were also found in their neutrophil granules (Mine and Wain, 1988; Wernersson *et al.*, 2006).

Inflammation is a combination of many cellular responses with the ultimate goal of pathogen clearance and/or tissue repair. Inflammation results in heat production, pain, redness and swelling. Cytokine secretion is also a major part of this process. Cytokines, such as IL1β and

TNFα, are heavily involved in promoting inflammatory responses and facilitating pathogen clearance. TNFα enhances the phagocytic potential of macrophages and primes the production of nitric oxide (NO) (reviewed by Grayfer and Belosevic, 2012). In mammals, TNFα associates with its receptors, TNFR1 and TNFR2, to mediate a wide variety of responses (reviewed by Parameswaran and Patial, 2010). TNFR1 is ubiquitously expressed in most tissues while TNFR2 is primarily expressed on immune cells, glial cells and some endothelial cells (reviewed by Medler and Wajant, 2019). Pfeffer et al. (1993) found that TNFR1 knockout mice were highly susceptible to *Listeria monocytogenes* infection due to inefficient clearing of bacteria. TNFR2 knockout mice, on the other hand, showed a marked decrease in TNF-induced tissue necrosis (Erickson *et al.*, 1994). Both receptors are suggested to have pro-inflammatory functions and have distinct, as well as overlapping, signalling mechanisms through which they mediate their immune functions (Kalb *et al.*, 1996). The role of TNFα was found to be evolutionarily conserved between mammals and fish when the first teleost TNFα transcript was discovered in Japanese flounder (Hirono *et al.*, 2000). The intron/exon organization was found to be similar to mammalian TNFα but it had only about 30% amino acid identity to mammalian TNFs. Additionally, there was a clear increase in expression levels of TNFα when fish were exposed to lipopolysaccharides (LPS), Phorbol 12-myristate 13-acetate (PMA) and concanavalin A (Con A), suggesting its role in fish inflammation. Following its discovery in flounder, TNFα was then discovered in many other fish species including zebrafish, channel catfish and rainbow trout (reviewed by Grayfer and Belosevic, 2012). Overall, the roles of TNFα in various teleost species seem to be functionally equivalent to mammalian TNFα.

IL1β triggers basophil histamine release, eosinophil degranulation, chemotaxis of B and T cells and it also induces the synthesis of other cytokines (reviewed by Grayfer and Belosevic, 2012). Similar to TNFα, IL1β is also evolutionarily conserved. The first IL1β transcript was identified in trout with 49-56% amino acid identity to mammalian IL1βs (Zou, 1999). In trout, a recombinant form of IL1β (rtIL-1β) was created to further test the role of this cytokine *in vivo* (Hong *et al.*, 2001). When rtIL-1β was intraperitoneally injected in trout, it resulted in increased resistance to *Aeromonas salmonicida* infection, increased the phagocytic response of peritoneal leukocytes and it also increased the expression of IL1β (Hong *et al.*, 2003). These functions of teleost IL1β supports its important role in fish inflammation.

IL8 is a specialized cytokine protein that activates chemotaxis, which is a process of immune cell migration towards infection sites. IL8, also known as CXCL8, is particularly important in neutrophil recruitment (reviewed by Havixbeck & Barreda, 2015). Additionally, CXCL8 is also expressed in teleost fishes and it seems to be functionally equivalent to mammalian CXCL8 (van der Aa *et al.*, 2010).

The complement system represents a complex innate network of antimicrobial proteins that are activated during infection. The complement system aids in phagocytosis and inflammation while also possessing the ability to attack the membranes of pathogens (reviewed by Muller-Eberhard, 1986; Sunyer and Lambris, 2001). There are three main pathways of the complement system. The classical pathway operates via binding of antibodies to foreign substances; the lectin pathway involves the binding of mannose-binding lectins (MBLs) to carbohydrate ligands on pathogen surfaces; and the alternate pathway, which involves the direct binding of complement component 3 (C3), to lipids, proteins and carbohydrates on

pathogen surfaces (Sarma and Ward, 2011; Afshar-Kharghan, 2017). One of the major functions of the complement system is the formation of the membrane attack complex (MAC). This is dependent on five main complement components; C5b, C6, C7, C8 and C9. These five components bind together and insert themselves into the plasma membrane of the pathogen. This forms a pore in the cell membrane and, therefore, results in cell lysis. Furthermore, the complement system also participates in chemotaxis through complement components, C3a and C5a (Sarma and Ward, 2011). They are also known as anaphylatoxins and use chemotaxis to attract innate immune cells to the sites of infection. Furthermore, complement component 3b (C3b) is a potent opsonin that coats foreign cells and essentially tags them for innate immune cells to phagocytose. As the name suggests, the complement system is not mutually exclusive but rather facilitates other innate immune functions. For example, a major mediator of the inflammatory response is IL8 which uses chemotaxis to recruit immune cells to the site of infection. C3a and C5a are complement components that serve the same purpose and therefore, enhance the inflammatory response. In teleosts, all the components of the complement system have been identified and were found to be homologous to the mammalian components (Boshra, Li and Sunyer, 2006).

Innate immune processes are heavily conserved across fish and mammals and the immunoregulatory receptors controlling these responses in mammals are also very well characterized, but the receptors that control innate immune responses in fish are largely unknown. In recent times, bioinformatics approaches have helped revolutionize the field of comparative immunology through the availability of genomes, transcriptomes and expressed sequence tag (EST) libraries. My research focuses on LITRs, a family of immunoregulatory

receptors. I will talk about these in greater detail in section 2.4 but first I will discuss immunoregulatory receptors in mammals and fish.

2.2 IMMUNOREGULATORY RECEPTORS IN MAMMALS

Immunoregulatory receptors are proteins that mediate the cellular effector responses (Barclay, 2003; Crocker, Paulson and Varki, 2007; Barrow and Trowsdale, 2008; Carlyle *et al.*, 2008; Flornes *et al.*, 2010). These receptors are usually expressed on cell surfaces. They contain extracellular domains that are critical for ligand binding. The ligands that bind these receptors range from features present on pathogens to unhealthy host cells. In other words, they can recognize PAMPs on foreign invaders to protect host tissues from pathogen infiltration. Furthermore, they can also recognize infected/injured host cells and can trigger cell effector responses in order to prevent these unhealthy host cells from affecting neighbouring cells and tissues. Generally, the extracellular regions of immunoregulatory receptors belong to either immunoglobulin superfamily (IgSF) or C-type lectin family. Here, I will only be focusing on IgSF proteins. Nonetheless, immunoregulatory receptors contain a transmembrane region that is mostly composed of hydrophobic amino acids to anchor the receptor to the cell membrane. Additionally, cytoplasmic tails are present to engage in signal transduction pathways. The general series of events that initiate an immune response is as follows: ligand binds the extracellular domains, this information is translated into specific intracellular signalling pathways which, in turn, result in the initiation of an immune response through elaborate recruitment and communication among signalling molecules. The focus of this section will be the well-characterized immunoregulatory receptors in mammalian systems.

In mammals, the immunoglobulin superfamily (IgSF) of receptors are a very large immunoregulatory protein family consisting of many receptors with the commonly shared feature of containing one or more immunoglobulin-like extracellular domains. Immunoglobulin (Ig) domains consist of two packed anti-parallel β-sheets. Additionally, Ig domains contain two highly conserved cysteines that form disulfide bridges that act to stabilize the structural conformation. This family is very well characterized in mammals and are known to be heavily involved in many immune functions. For example, the human leukocyte Ig-like receptors (LILRs) and killer Ig-like receptors (KIRs) are encoded by the human leukocyte receptor complex (LRC) on chromosome 19, whereas the fragment crystallizable receptors (FcRs) and Fc receptor-like (FcRLs) receptors are encoded by human chromosome 1 (Trowsdale, 2001; Davis, 2007; Carrillo-Bustamante, Keşmir and de Boer, 2016). Although all IgSF sub-families contain Ig domains, they actually bind different ligands and mediate various responses.

As the name suggests, Fc receptors bind the Fc region of antibodies. It is known that macrophages use Fc receptors to activate phagocytosis while NK cells use them for killing target cells using a process known as antibody-dependent cellular cytotoxicity (ADCC) (Takai, 2005; Gilfillan and Tkaczyk, 2006; Nimmerjahn and Ravetch, 2007). There are three classes of Fc receptors that are named after the antibody they bind: FcεR, binds IgE; FcγR, binds IgG; and FcαR, binds IgA. FcεRI is expressed on basophils and mast cells, and it is involved in regulating allergic reactions (Von Bubnoff *et al.*, 2003). There is a unique type of FcαR that binds IgM in addition to IgA, known as, Fcα/μR, and it is known to endocytose IgM coated microbes (Shibuya *et al.*, 2000). There are four types of FcγRs (FcγRI, FcγRII, FcγRIII, FcγRIV) owing to the fact that there are four different isotypes of IgG (reviewed by Nimmerjahn and Ravetch, 2008). FcγRs are

expressed on a wide array of immune cells and can activate responses such as phagocytosis, inflammation and NK cell mediated ADCC (DingYoung, Ko and Cohn, 1984; Titus *et al.*, 1987; Anderson *et al.*, 1990). FcRLs are very closely related to FcRs and are known to be an ancient multigene family (Davis, 2007). FcRLs 1-5 are expressed on B-cells while FcRL6 is expressed on NK cells. FcRL4 and FcRL5 have been implicated in regulating B-cell signalling through the binding of IgA and IgG, respectively (Wilson, Fuchs and Colonna, 2012). FcRL6, on the other hand, binds to major histocompatibility class II (MHC II) which suggests its role in differentiating between healthy and unhealthy host cells (Schreeder *et al.*, 2010).

KIRs are expressed on NK cells that mainly serve the purpose of mediating the killing of target cells or stressed host cells. All nucleated cells express major histocompatibility class I molecules (MHC I) (Trowsdale, 2001). However, a common aspect of viral infections is the downregulation of MHC I on host cells (Ishido *et al.*, 2000; Huang *et al.*, 2014; Koutsakos *et al.*, 2019). One reason why viruses have evolved this strategy is to evade T-cell detection. For example, cytotoxic T-cells (CTLs) mediate their functions via interactions with MHC-bound antigens (Hennecke and Wiley, 2001). In other words, CTLs recognize and bind MHC-I/antigen complexes which results in the initiation of its cytotoxic functions. When MHC is downregulated then this has a direct result on virus-infected host cells successfully evading CTL-mediated cytotoxicity. According to the missing-self hypothesis, NK cells are able to counter this strategy of viruses (Ljunggren and Kärre, 1990). This hypothesis suggests that missing “self” molecules (i.e. MHC molecules) on host cells will result in their killing by NK cells via KIRs. If KIRs do not detect MHC on host cells then NK cell mediated cytotoxicity is triggered. These receptors are unique to the mammalian species but functional relatives have been identified in birds (i.e.

chicken Ig-like receptors, CHIRs), fish (i.e. *Ictalurus punctatus* leukocyte immune-type receptors, IpLITRs) and amphibians (i.e. *Xenopus* MHC-linked Ig superfamily V genes, XMIV) (Ohta *et al.*, 2006; Stafford *et al.*, 2006; Straub *et al.*, 2013)

Immunoregulatory receptors are involved in activating immune responses but are also involved in inhibiting responses (Kelley, Walter and Trowsdale, 2005; Barrow and Trowsdale, 2008). They are co-expressed on immune cells and work to facilitate the proper functioning of the immune system. This stimulatory-inhibitory regulation is key to prevent the development of autoimmune diseases and to prevent infections. Over the course of evolutionary history, the signalling transduction mechanisms have been shown to be conserved. More specifically, the presence of tyrosine-based motifs are conserved in mammalian and non-mammalian models that essentially mediate cell effector functions. Due to their conserved nature and important roles in regulating immune responses, stimulatory and inhibitory tyrosine-based motifs will be discussed briefly below.

2.2.1 Tyrosine-based stimulatory and inhibitory signalling

Stimulatory receptors usually have a very short cytoplasmic tail (CYT) and a positively charged transmembrane (TM) region (i.e. they contain an arginine (R) or lysine (K) residue). This allows these receptors to bind to the negatively charged TM segments of intracellular adaptor signalling molecules which mediates their functional responses (Feng, Call and Wucherpfennig, 2006). For example, adaptor proteins usually have one or more tyrosine-based motifs, known as immunoreceptor tyrosine-based activating motifs (ITAM), encoded within their CYT regions. There are exceptions to this criteria as FcγRIIA, a platelet receptor, has an ITAM embedded in its CYT region, therefore it does not need to associate with adaptor molecules to initiate

signalling (reviewed by Hamerman *et al.*, 2009). ITAMs are responsible for activating a number of immune responses such as cytokine secretion and phagocytosis (Biassoni *et al.*, 2000; Treichel *et al.*, 2004; Vivier, Nunès and Vély, 2004; Nimmerjahn and Ravetch, 2006). When ITAMs were first discovered, the general consensus for this motif was D/ExxYxxL/I(x_{6-8})YxxL/I (where x can be any amino acid; Reth, 1989). Common examples of intracellular adaptor proteins that contain ITAMs includes the DNAX-activating protein 12 (DAP12) and the FcRγ chain, which are found in most innate immune cells, including granulocytes, NK cells, mast cells and dendritic cells (Hamerman *et al.*, 2009). In humans, DAP12 associates with CD94/NKG2C and FcRγ associates with Fcγ and Fcε receptors.

Inhibitory receptors classically contain one or more tyrosine based motifs in their long CYT regions (Vivier and Daëron, 1997; Ravetch and Lanier, 2000). The general consensus of an immunoreceptor tyrosine-based activating motif (ITIM) is S/I/V/LxYxxI/V/L (Ravetch and Lanier, 2000). Contrary to some stimulatory receptors, inhibitory receptors do not need to associate with adaptor proteins as their CYT region can initiate inhibitory responses directly via their ITIMs. KIRs are classic examples of inhibitory receptor-types. When they were first discovered, they were actually termed killer inhibitory receptors but later changed to killer immunoglobulin-like receptors due to the fact that some activating KIRs also exist (reviewed by Pende et al., 2019). In general, ITIMs inhibit stimulatory receptors by recruiting Src homology 2 (SH2)-containing tyrosine phosphatases such as SH2-containing inositol phosphatase (SHIP), the SH2-containing protein tyrosine phosphatase 1 and 2 (SHP-1, SHP-2) (Healy and Goodnow, 1998; Long, 1998). Phosphatases remove phosphate groups from phosphorylated molecules

(i.e. dephosphorylate targets) which subsequently blocks any further kinase signalling. This results in the inhibition of effector responses.

There is another tyrosine-based motif known as immunoreceptor tyrosine-based switch motif (ITSM). The consensus sequence for this is TxYxxV/I and it was discovered based on its ability to bind the adaptor protein, Src homology 2 domain-containing molecule 1A (SH2D1A) (Shlapatska *et al.*, 2001; Sidorenko and Clark, 2003). This motif is unique because it can participate in both stimulatory and inhibitory pathways depending on which immune cell it is expressed in. For example, CD244 (also known as 2B4), is expressed in NK cells, monocytes, basophils, and some T cells (Romero *et al.*, 2004). There are also two isoforms of murine 2B4; the long 2B4 variant (2B4L) has four ITSMs in its CYT region while short splice variant (2B4S) has only one ITSM (Stepp *et al.*, 1999). It was shown, in NK cells, that 2B4L inhibited tumor lysis while 2B4S stimulated the NK cell-mediated killing (Schatzle *et al.*, 1999).

Both stimulatory and inhibitory receptors are expressed on immune cell surfaces in mammalian and non-mammalian models. The stimulatory-inhibitory paradigm is crucial for the proper functioning of the host immune system. I will now focus on the immunoregulatory receptor families in teleosts to further reiterate the conserved nature of tyrosine-based motifs and their importance in regulating in teleost immunity.

2.3 IMMUNOREGULATORY RECEPTORS IN TELEOST

As mentioned earlier, recent advancements in bioinformatics made it possible to identify various novel receptor genes in teleost. In this section, I will only briefly talk about some immunoregulatory receptor-types that have been identified in teleost, then I will focus on IpLITRs in section 2.4.

2.3.1 Examples of Immunoglobulin receptors in fish

The only homolog of FcR to be identified in teleost is the IpFcRI (Stafford *et al.*, 2006). It was found in NK-like cells of channel catfish. The discovery of this receptor was motivated by the presence of IgM antibodies binding to the surface of these cells (Shen *et al.*, 2003, 2004). The researchers hypothesized the possible presence of a putative FcμR. They observed that when native IgM (from serum) was replaced with catfish anti-trinitrol-phenol (TNP) IgM antibodies, the NK-like cells were able kill TNP opsonized targets via ADCC (Shen *et al.*, 2003). This strongly suggested the presence of a putative functional FcμR in fish. This inspired further bioinformatics searches, mainly via EST databases, to find relatives of mammalian FcRs in catfish, which resulted in the discovery of the IpFcRI gene. This catfish immunoregulatory receptor has three Ig domains but no TM or CYT regions which led to the possibility of it being soluble or intracellularly expressed. Stafford et al. (2006) tested this theory by transfecting native IpFcRI into insect cells and found that IpFcRI was present in the cell supernatants via western blot. Additionally, IpFcRI is heavily expressed in lymphoid tissues and was detected in its native state in catfish plasma. This suggests that IpFcRI is a secreted protein, but its function *in vivo* remains unknown although it is likely a soluble IgM-binding protein.

Novel immune-type receptors (NITRs) were first characterized in Southern pufferfish (Rast *et al.*, 1995). Since their discovery, NITRs have been identified in 14 other teleost species including zebrafish and channel catfish (Ferraresso *et al.*, 2009; Yoder *et al.*, 2010). NITRs contain extracellular Ig domains that are related to T-cell receptors, however they are considered to be functional orthologs of KIRs (Litman, Hawke and Yoder, 2001; Yoder, 2009). These receptors have been identified to have stimulatory and inhibitory types (Yoder *et al.*,

2008; Rodríguez-Nunez *et al.*, 2014). In zebrafish, all NITRs are found to be expressed in lymphoid tissue which further supports their designation as NK receptors (NKRs; Yoder et al., 2010). Furthermore, in zebrafish, NITRs only seem to be present in adult tissues, with the exception of *nitr3* family, which are ubiquitously expressed throughout ontogeny and adulthood. NITRs are exclusive to teleost while IpLITRs are distantly related to several immunoregulatory receptors in other mammals including KIRs, LILRs, FcRs and FcRLs (Stafford *et al.*, 2006).

2.4 TELEOSTS AS MODEL ORGANISMS TO UNDERSTAND IMMUNITY

Teleost are a very large group consisting of about 32,000 species, representing the largest group of vertebrates (reviewed by Wilson, 2017). They have adapted to various aquatic conditions and are constantly exposed to a wide variety of pathogens. The innate immune systems of mammals and bony fish have lots of similarities suggesting the evolutionarily conserved nature of various protective mechanisms. As mentioned earlier, functional homologs of various mammalian innate immune components have also been discovered in teleost.

2.4.1 Overview of catfish as a model organism

Channel catfish are unique because there are isolated long-term leukocytes that can be maintained in culture without the need for transformation (Miller *et al.*, 1998). There are many types of clonal immune cells that are available including B-cells, T-cells, macrophages and NK-like cells. For example, 3B11 is a B cell line generated from an outbred catfish that was stimulated by mitogens (Wilson *et al.*, 1997). Cytotoxic T-cells, TS32.15 and TS32.17, were developed from catfish mixed leukocyte reactions (MLC) that were immunized with irradiated B-cells (Stuge *et al.*, 2000). NK-like cells, 1F3, was developed by stimulating peripheral blood

leukocytes (PBLs) with irradiated B-cells (Shen *et al.*, 2004). Macrophage cell line, 42TA, was acquired from the blood of an outbred catfish (Miller, Chinchar and Clem, 1994). Stafford et al. (2006) took advantage of the availability of these catfish cell lines to identify and characterize a novel family of IgSF receptors called IpLITRs.

2.4.2 Discovery of Leukocyte Immune-type Receptors (IpLITRs)

EST libraries generated from various cell lines were used to search for teleost immunoregulatory receptor-type genes. As a result, three IpLITRs were originally identified (i.e. IpLITR1, IpLITR2 and IpLITR3). These receptors were shown to be expressed in various tissues, such as spleen, gill and kidney. They are also expressed in all immune cells examined, such as macrophages, NK-like cells, T-cells and B-cells. Additionally, IpLITRs were not expressed in a non-immune catfish ovarian cell line suggesting that these receptors may be highly involved in immunological processes. IpLITR1, IpLITR2 and IpLITR3 have four, three and six Ig domains, respectively. Additionally, IpLITR1 has a CYT region consisting of two ITIMs, one ITSM and one ITIM-like motif (SEYTTE). IpLITR2 and IpLITR3 have positively charged TM regions, due to the presence of lysine, K, or arginine, R, with no tyrosine-based motifs in their relatively short CYT regions. This suggests that IpLITR1 is a putative inhibitory receptor while IpLITR2 and IpLITR3 are putative stimulatory receptor-types. Interestingly, there is also a high variability among Ig domains between the receptors. For example, Ig domains, D1 and D2, are highly similar to one another while D3 of IpLITR2 is only about 17-39% similar to other domains. IpLITR3 D5 and D6 domains are only about 15-25% similar to other IpLITR Ig domains. When compared to mammalian sequences, IpLITRs are distantly related to FcRLs, KIRs and LILRs. More specifically, D1 and D2 are related to FcRLs while D3 and D4 domains are related to the LRC-encoded

receptor Ig domains. EST databases were also used to screen for putative LITR-types in zebrafish and it was shown that the D1 and D2 domains of IpLITRs are not at all related to zebrafish LITR-like sequences. Interestingly, LITRs are not closely related to other identified teleost receptors, such as NITRs. This solidified LITRs as a novel family of IgSF receptors within teleost.

The function of LITRs *in vivo* are not known (Stafford *et al.*, 2007). When catfish PBLs were stimulated with LPS and con A, IpLITRs were not expressed for a period of 12 days. On the contrary, when PBLs were stimulated with alloantigen (e.g. irradiated catfish B cells), IpLITR1 and IpLITR2 were co-expressed at day 8 and co-expressed again at days 2, 4, 6 and 8 after re-stimulation. Additionally, alloantigen stimulation induced the expression of many related IpLITR-like genes. When these transcripts were cloned and sequenced, about forty new LITR-types were identified from the original three prototype IpLITRs identified. All of these transcripts encode extracellular domains of variable lengths as well as CYT lengths.

To summarize, the function of IpLITRs is not known but there is evidence that they may be participating in alloantigen-induced immunity. The use of catfish clonal cell lines is a very powerful tool as it has led to the discovery of a novel immunoregulatory family in teleost. However, the regulatory functions of these receptors are still not known, and their signalling mechanisms are largely uncharacterized. Therefore, the Stafford lab has used heterologous expression systems to further examine the functional potential of IpLITRs and this will be the main focus of the next two sections.

2.5 STIMULATORY IpLITR-TYPES

2.5.1 Recruitment of adaptor proteins

As mentioned earlier, alloantigen stimulation resulted in the generation of additional LITR-like proteins (Stafford *et al.*, 2007). One of these variants was IpLITR 2.6b which is an isoform of IpLITR2. IpLITR 2.6b is considered a putative stimulatory receptor due to the presence of a charged TM region (lysine, K) and a short CYT region devoid of any tyrosine-based motifs. In mammalian systems, stimulatory receptors, devoid of CYT regions, associate with adaptor proteins with signalling motifs to initiate an immune response (Hamerman *et al.*, 2009). Mewes et al. (2009) hypothesized that IpLITR 2.6b will also form associations with adaptor proteins, as seen with mammalian systems. To test this hypothesis, IpLITR 2.6b was transfected into human embryonic kidney (HEK 293T) cells with the addition of an N-terminal hemagglutinin (HA) epitope. Additionally, C-terminal FLAG-tagged adaptor proteins IpFcRγ, IpFcRγ-like(L), IpCD3ζ-L, and IpDAP12, with negatively charged (aspartic acid, D) TM segments, were co-transfected with the HA-tagged IpLITR 2.6b. All of these adaptors have ITAM-containing CYT regions. Using this approach, it was shown that the positively charged IpLITR 2.6b associates with the negatively charged IpFcRγ, IpFcRγ-like(L) and IpCD3ζ-L. Additionally, interactions with IpFcRγ and IpFcRγL increased cell surface expression of the receptor while no differences in surface expression were observed with the other adaptor proteins. Interestingly, IpLITR 2.6b did not associate with DAP12. This was surprising because in mammalian systems, DAP12 is one of the most common adaptor proteins utilized by members of the IgSF family (Hamerman *et al.*, 2009). Also, in mammals, receptors containing a lysine residue, like IpLITR 2.6b, mostly associate with DAP12 (Feng, Call and Wucherpfennig, 2006). Mewes et al. (2009)

tested to see if the type of residue in the TM region affects receptor-adaptor interactions. They mutated the lysine in the TM region to a positively-charged arginine or an uncharged alanine. They also mutated the aspartic acid of IpFcRγL to an alanine. Surprisingly, the mutation in the TM region of the receptor had no effect on its binding affinity. This suggested that the TM region of IpLITR 2.6b does not have to be charged to associate with adaptors. The complete opposite effect was seen for the adaptor, IpFcRγL. Without the negative charge, the function of the adaptor was completely lost, and it could not associate with the receptor. This suggested that the negative charge is crucial for the adaptor protein to maintain its function. Additionally, stimulatory IpLITRs have the ability to form non-covalent homo- and heterodimers through predicted interactions among their Ig domains. To summarize, IpLITR 2.6b associates with ITAM-containing adaptor proteins and therefore, has the ability to potentially stimulate immune responses.

To further examine the role of IpLITR 2.6b-mediated effector functions, Cortes et al. (2012) fused the two extracellular domains of IpLITR 2.6b to the TM and CYT regions of IpFcRγ-L to create the chimeric protein, called IpLITR2.6b/IpFcRγ-L. This chimeric protein has an N-terminal HA epitope and will be referred to as IpLITR 2.6b, for the rest of this section. In this study, the researchers used a new cell line, rat basophilic leukemia cells (RBL-2H3), as they wanted to test immune processes in established immune cells. The chimeric IpLITR 2.6b was transfected into RBLs and the arginine residue in the TM of IpFcRγ-L was mutated to alanine to prevent possible interactions with any RBL-2H3 cell endogenous molecules. Additionally, they created a construct where the dual tyrosine residues of ITAM were mutated to phenylalanine (F). This construct was named IpLITR/IpFcRγ-L FF. After stimulating IpLITR 2.6b with the anti-HA

mouse antibody (mAb), IpLITR 2.6b induced mast cell degranulation. This stimulatory effect was lost in IpLITR/IpFcRγ-L FF suggesting the importance of a functional ITAM for the initiation of the response. Cortes et al. (2014) also found that IpLITR 2.6b was capable of triggering the secretion of IL3, IL4, IL6 and TNFα in transfected RBLs. Furthermore, when RBL-2H3 cells expressing IpLITR 2.6b were incubated with αHA coated 4.5μm polystyrene beads, the cells performed ITAM-dependent phagocytosis of the opsonized targets. This showed for the first time that LITRs have the ability to trigger the phagocytic response in innate immune cells.

ITAM-dependent signalling in mammalian FcRs is very well-characterized (reviewed by Getahun and Cambier, 2015). Once a signalling cascade is initiated, many signalling molecules are recruited to the activated receptor to facilitate an immune response. Lillico et al. (2015) blocked some important signalling molecules in the classically described ITAM-mediated pathway using pharmacological inhibitors to see if phagocytosis was affected in IpLITR-expressing RBL-2H3 cells. The inhibitors used and their target molecules were: actin polymerization (Latrunculin B), protein kinase C (PKC; Go6976), spleen tyrosine kinase (Syk; ER 27391), protein kinase B (also known as, Akt; Akt inhibitor VIII), phosphatidylinositol 3-kinase (PI3K; Wortmannin), c-Src tyrosine kinases (KB SRC4 and PP2), Cdc42 (ML 141), Rac1/2/3 (EHT 1864), phosphoinositide-dependent kinase 1 (PDK1; GSK 2334470), and mitogen-activated protein kinase (MEK1/2; U0126). Blockage of these signalling molecules caused variable effects on the inhibition of IpLITR-mediated phagocytosis suggesting that IpLITR 2.6b may utilise the same ITAM-dependent pathway as mammals. Furthermore, confocal imaging showed that IpLITR 2.6b mediates phagocytosis via a phenotype previously described in mammals (Levin, Grinstein and Schlam, 2015; Lillico *et al.*, 2015). This includes target binding, extension of

membrane (pseudopod) and engulfment. This suggests that the ITAM-mediated signalling pathway is evolutionarily conserved between mammals and fish. To summarize, IpLITR 2.6b was shown to stimulate the immunological processes of phagocytosis, degranulation and cytokine secretion (Cortes *et al.*, 2012, 2014; Lillico *et al.*, 2015).

2.6 INHIBITORY IpLITR-TYPES

2.6.1 Inhibition of NK cell-mediated cytotoxicity

Montgomery et al. (2009) tested to see if the ITIM-containing CYT regions of IpLITRs can perform inhibitory functions. The IpLITRs that were chosen for testing were IpLITR 1.1b and 1.2a as both have ITIMs in their tail (Stafford *et al.*, 2007). Specifically, the CYT region of IpLITR 1.1b contains membrane proximal and membrane distal regions. Each region encodes three tyrosine residues (Proximal: Y_{433}, Y_{453} and Y_{463}; Distal: Y_{477}, Y_{499} and Y_{503}). The distal section encodes one ITSM (Y_{503}) and two ITIMs (Y_{477}, Y_{499}). IpLITR 1.2a is similar to 1.1b except it doesn't have a proximal CYT region and has only one ITIM and one ITSM. The CYT regions of these two IpLITRs (1.1b and 1.2a) were fused to the human NK-cell receptor, KIR2DL3. This receptor was chosen because the ligand (HLA-Cw3) is known and an anti-KIR2D antibody (DX27) is available (Montgomery *et al.*, 2009). In other words, the extracellular domains are from KIR2DL3 while the signalling capacity (i.e. CYT region) are provided from the IpLITRs. The results showed that IpLITRs recruit SHP-1 and SHP-2 to their cytoplasmic tails, similar to mammalian ITIM-containing tails (Healy and Goodnow, 1998; Long, 1998; Montgomery *et al.*, 2009). Using the same chimeric constructs, Montgomery et al. (2012) tested to see if IpLITRs can also inhibit a functional response *in vitro*. This study showed that IpLITR 1.1b and 1.2a actively participated in the inhibition of mouse NK cell-mediated cytotoxicity. Additionally, it was found that IpLITRs

can inhibit a response via SHP-dependent and SHP-independent mechanisms. The unique SHP-independent mechanism included the recruitment of the inhibitory c-src kinase (Csk) to the tyrosine-containing proximal region of IpLITR 1.1b. In mammalian systems, Csk phosphorylates src family kinases (SFK) at regulatory tyrosine residues, which results in the structural inactivation of kinases (Okada *et al.*, 1991). To summarize, Montgomery et al. (2012) showed that the inhibition of NK cell-mediated cytotoxicity was regulated differentially and independently by the proximal and distal CYT regions of IpLITR 1.1b using both SHP-independent and SHP-dependent mechanisms, respectively.

2.6.2 Induction of phagocytosis in RBL-2H3 cells

To further investigate IpLITR 1.1b-mediated immune processes, Cortes et al. (2014) stably expressed IpLITR 1.1b in RBL-2H3 cells. They showed that IpLITR 1.1b induced the phosphorylation of extracellular signal–regulated kinases 1 and 2 (ERK1/2) and protein kinase B (also known as, Akt). These two kinases have been known to be involved in activation of processes, such as cell proliferation (Asati, Mahapatra and Bharti, 2016). Unexpectedly, this suggested that IpLITR 1.1b, an ITIM-containing inhibitory receptor (described above) was also capable of stimulating cellular responses. Specifically, IpLITR 1.1b induced phagocytosis via its ITIM-containing CYT region (Cortes *et al.*, 2014). The researchers removed the signalling capacity of the receptor (i.e. removed the cytoplasmic tail) and found that phagocytosis was inhibited. Also, IpLITR 1.1b-mediated phagocytosis is actin-dependent as treatment with Cytochalasin D (inhibitor of actin polymerization) abrogated the phagocytic response. Additionally, IpLITR 1.2a was also triggered the phagocytic response in transfected RBL-2H3 cells. This suggests that an ITIM and ITSM in the distal region may be involved in the unique

stimulatory potential of the receptor. ITSMs have been implicated in both stimulatory and inhibitory function so it is possible that it might be mediating this unexpected IpLITR 1.1b-mediated stimulatory response (Schatzle *et al.*, 1999). Cortes et al. (2014) did not carefully study the role of the cytoplasmic tail of IpLITR 1.1b. However, Montgomery et al. (2012) showed that the distal and proximal regions of the cytoplasmic tail of IpLITR 1.1b were capable of inhibiting NK cell-mediated cytotoxicity independent of one another. These results, when combined, suggest that this receptor demonstrates functional plasticity (context dependent signalling) and is able to mediate both stimulatory and inhibitory responses using unknown novel mechanisms.

To further investigate these mechanisms, Lillico et al. (2015) used a pharmacological approach to identify potential signalling molecules that might be involved in IpLITR 1.1b-mediated phagocytosis. The classical mammalian ITAM-mediated phagocytic pathway involves many intracellular molecules (Getahun and Cambier, 2015). The process begins with the phosphorylation of tyrosines by a member of src-family tyrosine kinases which, in turn, recruit Syk. This kinase then mediates phosphorylation of other signalling molecules to activate them. These important signalling molecules include PI3K, guanine exchange factors (GEFs, e.g. Vav) and small Rho GTPases (e.g. Cdc42 and Rac1). When these molecules were inhibited using known pharmacological drugs, IpLITR 1.1b was surprisingly still able to induce phagocytosis (Lillico *et al.*, 2015). Phagocytosis is also an actin-dependent process so when actin polymerization was inhibited, IpLITR 1.1b was unable to mediate phagocytosis. The same result was seen when src-family kinases and Syk were inhibited. IpLITR 2.6b-mediated phagocytosis was also essentially “shut down” when these key molecules were inhibited. These results

suggest that IpLITR 1.1b uses a different signalling mechanism but is still dependent on actin, src-family tyrosine kinases and Syk for the initiation of its unique phagocytic response.

IpLITR 1.1b was later found to also trigger exhibit a novel phagocytic phenotype, as visualized by confocal microscopy (Lillico *et al.*, 2015). The classical phagocytic phenotype involves binding of the ligand, pseudopod extension and engulfment (Flannagan, Jaumouillé and Grinstein, 2012). IpLITR 2.6b followed this criteria very closely (Lillico *et al.*, 2015). IpLITR 1.1b, on the other hand, displayed a phenotype wherein targets were bound to the membrane but not engulfed rapidly, as seen with IpLITR 2.6b. Additionally, IpLITR 1.1b triggered the formation of finger-like membrane extensions that tethered the targets to the surface and there were more targets tethered to the surface of the membrane than actually engulfed. To summarize, the fact that an ITIM-containing IpLITR 1.1b can stimulate actin polymerization but can also inhibit cytotoxicity, suggests that this receptor is versatile in its functional capabilities. Furthermore, the differential recruitment of signalling molecules to its CYT region highlights its functional plasticity as it is able to mediate the same response using either section of its cytoplasmic tail.

2.6.3 Proposed signalling mechanisms for IpLITR 1.1b-mediated functional plasticity

The recent findings about the unique phagocytic phenotype of IpLITR 1.1b led to the questioning of the possible signalling mechanisms of this receptor. Fei et al. (2016) used published literature and Basic Local Alignment Search Tool (BLAST) searches to propose some signalling mechanism by which IpLITR 1.1b mediates immune functions.

IpLITR 1.1b can inhibit NK cell-mediated cytotoxicity through its proximal and distal regions independently (Montgomery *et al.*, 2012). Pils et al. (2012) found that in human

carcinoembryonic antigen-related cell adhesion molecule 3 (CEACAM 3), a member of IgSF, Wiskott-Aldrich syndrome protein family member 2 (WAVE2) complex is activated via the recruitment of non-catalytic region of tyrosine kinase adaptor protein (Nck) to the CYT region. These events are involved in the promotion of actin-dependent membrane remodeling. Fei et al. (2016) proposed that this Nck/WAVE2 mechanism may also be utilised by IpLITR 1.1b as it contains a consensus sequence, HIYDEV, found in its proximal CYT region (Montgomery *et al.*, 2009). This sequence has been shown to recruit Nck, therefore, it may be implicated in IpLITR 1.1b-mediated phagocytosis (Frese *et al.*, 2006). The distal region, on the other hand, may uniquely recruit Syk in an ITAM-independent manner (Fei *et al.*, 2016). In platelet endothelial cell adhesion molecule-1 (PECAM-1), it has been shown that Syk is recruited to the two ITIM-containing CYT region spaced apart by 22 amino acids (Wang *et al.*, 2011). Coincidentally, this is the exact same length of separation between the two ITIM tyrosine residues in the IpLITR 1.1b distal CYT region, (Stafford *et al.*, 2007). The recruitment of Syk can lead to the activation of GEFs, such as Vavs, and Rho GTPases, which can then lead to actin polymerization (Fei *et al.*, 2016).

To test this model, Zwozdesky et al. (2017) used a biochemical approach. They fused glutathione S-transferase (GST) to the CYT region of IpLITR 1.1b. The GST-CYT construct was incubated with human epithelial cell (AD293) lysates expressing various FLAG-tagged SH2-domain containing target proteins (i.e. SHP-2, Csk, Syk, Grb2, Nck1, PI3K, p85α, Vav1 and Vav3). This GST-CYT construct acted as “bait” to bind the FLAG-tagged SH2-domain containing proteins as “prey”. This technique then allows for the specific detection of various protein interactions via immunoprecipitations. The full-length CYT region of IpLITR 1.1b associated with SHP-2, Csk,

Syk, Grb2, Nck1, PI3K p85α, and Vav1. The proximal region associated with Csk, Grb2, Nck1, PI3K, p85α and Vav1, and the distal region associated with SHP-2, Syk, PI3K p85α. This suggests that these molecules are potentially involved in IpLITR 1.1b-mediated phagocytosis.

Lillico et al. (2019) investigated the unique "finger-like membrane extensions", known as filipodia, that IpLITR 1.1b displays when stimulated with targets. They focused on Nck and phosphorylated Syk (pSyk) molecules to see if these two key effector proteins are involved in the generation of these structures. They incubated the IpLITR 1.1b-expressing RBL-2H3 cells with αHA mAb opsonized targets and these were stained with a fluorescent secondary antibody which can be visualized using confocal microscopy. It was found that endogenous Nck co-localizes with IpLITR 1.1b within filipodia structures but pSyk did not. Additionally, it has been shown that Nck and pSyk are potentially involved in all stages of the IpLITR 1.1b-mediated phagocytic process: target binding, capture/pseudopod formation and engulfment. IpLITR 2.6b, on the other hand, was found to interact with Nck and pSyk mostly during the formation of the phagocytic cup, while a significant reduction is seen immediately after engulfment.

IpLITR 1.1b is considered to be "functionally plastic" as it has the ability to stimulate and inhibit responses. It can differentially mediate immune responses using its proximal and distal regions independently in both immune and non-immune mammalian cell lines. This supports the notion that non-mammalian models can be used to further examine the unique mechanisms of immunoregulatory receptor function. This will serve to broaden our understanding of the dynamic aspects of vertebrate intracellular signalling mechanisms.

2.7 Conclusions

IpLITRs are a unique immunoregulator receptor family originally discovered in the channel catfish that are phylogenetically and structurally related to several mammalian receptor-types, such as KIRs, LILRs and FcRs. IpLITRs are expressed on many immune cell types including macrophages and NK-like cells (Stafford *et al.,* 2006) and they appear to be involved in immune functions including phagocytosis, cytokine secretion, degranulation and the inhibition of NK cell-mediated cytotoxicity (Cortes *et al.*, 2012, 2014; Montgomery *et al.*, 2012). The utility of *in vitro* systems is invaluable as it is an excellent way to investigate the functional capabilities of receptors and is also very economically feasible. However, these *in vivo* results cannot be directly extended into what may occur *in vivo*. For example, IpLITR 2.6b is capable of stimulating phagocytosis in RBL-2H3 cells but it cannot be assumed that this receptor is involved in this process in a channel catfish facing a pathogen challenge. It is impossible to extend *in vitro* results into *in vivo* systems. The only way to combat this limitation is to move the characterization of LITRs to *in vivo* systems.

LITRs were discovered in catfish as a result of the availability of long-term leukocyte cell lines. This was a major advantage to using channel catfish as a model organism. However, there are certain limitations to this organism as well. When LITRs were initially discovered, the catfish genome was not available. Therefore, the chromosome locations of the characterized LITRs were not known and it was unknown if isoforms/splice variants were produced post alloantigen stimulation (Stafford *et al.*, 2007). As a result, there is currently no data available highlighting the genetic location of LITRs and there is no information on the number of LITRs expressed by catfish. Additionally, all LITR information has been only ever been obtained from adult catfish. It

is unknown if LITRs are present during ontogeny or if they are solely an adult-specific receptor system. The presence/absence of immune genes during development can answer new questions about their importance during embryogenesis and their role in immune defense.

To begin to understand the potential *in vivo* functions of fish LITRs, it is imperative to first identify some LITR-types in zebrafish, a fish species with a fully sequences and annotated genome. For my thesis project, I have decided to identify putative DrLITRs (*Danio rerio* leukocyte immune-type receptors) and track their gene expression levels throughout ontogeny and adulthood. Zebrafish are a great immunological model organism as well as a great developmental model (reviewed by Yoder *et al.*, 2002; Traver *et al.*, 2003). Fish have all the major immune cell lineages as mammals but zebrafish, specifically, offer additional advantages. In addition to a sequenced genome, they have very rapid life cycles relative to other teleost species and they can be very easily bred making it easy to monitor the developmental stages (Yoder *et al.*, 2002). Throughout development, there are key times where certain immune genes turn on and this has been well-characterized in zebrafish (Willett *et al.*, 1997, 1999, 2001; Trede, Zapata and Zon, 2001; Danilova and Steiner, 2002; Lam *et al.*, 2002, 2004). It is still unknown if immune genes are turned on early during development for the sole purpose of protection for the newborn or for aiding the development process. The role of LITR-types in development are unknown and the quantitative expression of these receptors during ontogeny and adulthood has never been tracked. Answering these questions can help us broaden our understanding of the role of the immunoregulatory receptors during teleost development and help us further understand the complexity and evolution of the vertebrate innate immune system.

CHAPTER III

MATERIALS AND METHODS

3.1 IDENTIFICATION OF IpLITR-RELATED SEQUENCES IN ZEBRAFISH

IpLITR 1, 1.1b, 2, 2.2a, 2.2b and 3 were used to seed the BLAST protein (BLASTp) searches in the Ensembl database (https://uswest.ensembl.org/index.html) (Zwozdesky, unpublished data). The Ensembl database was used because it integrates many other databases and annotates genomes using the latest available data. The top hits were compiled into a list and used to seed BLASTp searches against the zebrafish genome available on the Ensembl database. These top hits were also saved and added to the list. Furthermore, these sequences were analyzed to ensure the presence of immunoglobulin domains and transmembrane regions using SMART (http://smart.embl.de) and SOSUI (http://harrier.nagahama-i-bio.ac.jp/sosui/sosui_submit.html), respectively. In both of these databases, the putative DrLITR protein sequence is inserted. The SMART database predicts Ig domains and TM regions using phylogenetic relationships, functionally conserved residues and tertiary protein structures (Letunic and Bork, 2018). The transmembrane regions span the membrane so they are characterized by their hydrophobic nature. SOSUI analyzes sequences for large hydrophobic stretches so I used it to double check TM regions. Additionally, the stimulatory and inhibitory potentials of the CYT were also annotated based on conserved tyrosine-based motifs. I chose to analyze four potential DrLITRs based on their unique CYT regions (i.e. their unique signalling capabilities). I would use these putative DrLITRs sequences to seed Position Specific Iterative (PSI) BLAST searches against the catfish genome to account for distant evolutionary

relationships. If the top 200 hits contained IpLITRs then I would proceed with the *in vivo* characterization.

3.2 ANIMAL MAINTENANCE

The adult wild-type Tupfel Long fin (TL) zebrafish were maintained at the University of Alberta Aquatics Facility. All protocols, experimental procedures and fish housing were approved by the Animal Care and Use Committee at the University of Alberta (AUP #00000816) and performed in compliance with the guidelines of Canadian Council on Animal Care.

3.3 EMBRYOS

For the acquisition of embryos, three adult fish (two females and one male) were kept in breeding tanks overnight separated by a barrier between the two sexes. At 8 a.m., the barriers were removed allowing the fish to mate for 30-40 minutes. The embryos were then collected and reared in egg water (60 mg/L Instant Ocean). They were incubated at 28.5°C and the egg water was replenished daily. The embryos were sacrificed at specific time points by immersion in 500 µL of TriZol (Invitrogen # 15596026). The experimental time points chosen for embryos, in hours post fertilization (hpf), are: 0 (unfertilized eggs), 1, 6, 24, 48, 72, 96, 120, 144 and 168. Unfertilized eggs were acquired by anaesthetizing female fish in 100 mg/L of tricaine (MS-222) (Sigma-Aldrich) and applying gentle pressure on the ventral side of the fish to facilitate the release of eggs. Each experiment consisted of 50 embryos per time point. This experiment had a total of three replicates.

3.4 ZYMOSAN A EXPOSURES

Adult zebrafish were anaesthetized in 100 mg/L of tricaine (MS-222) (Sigma-Aldrich). A total of thirty-three fish were used in each experiment. For example, fifteen fish were intraperitoneally injected with 20 µL of 1X phosphate buffered saline (PBS) and fifteen fish were injected with 20 µL of zymosan A (1 mg/mL) (Sigma- Aldrich # 58856-93-2). The fish were then returned to their tanks and dissected at specific time intervals. The experimental time points chosen, in hours, were: 3, 6, 8, 12 and 24. Fish were immersed and euthanized in 200 mg/L of tricaine followed by decapitation. The viscera of the fish were obtained which contains the liver, intestines, pancreas and spleen of the fish. Three additional fish were injected with 1X PBS and sacrificed immediately to serve as the reference sample. One experimental replicate contained three fish each per time point and per treatment. This experiment had a total of three replicates.

3.5 RNA EXTRACTION

The isolated viscera from adult fish and embryos were submerged in 500 µL of TRIzol (Invitrogen # 15596026) and homogenized using a pestle motor mixer. RNA was extracted according to the manufacturer's instructions. The final RNA was treated with DNase I (Invitrogen #18068015) as per manufacturer's instructions. RNA concentration was determined by a NanoDrop 1000 Spectrophotometer (ThermoFisher). The 260/280 and 260/230 ratios were over 1.8 for all samples because low 260/280 and 260/230 ratios indicate protein contamination and residual phenol contamination, respectively. If ratios did not meet this standard, an ethanol precipitation protocol was performed to increase the quality of the RNA. The process started with adding 1/10 volume of Sodium Acetate, pH 5.2, to the dissolved RNA.

Pure ethanol (100%) was added to the solution so that the total concentration of ethanol does not decrease below 70%. The solution was vortexed and incubated at -80°C for 20 minutes and centrifuged at 8000xg for 20 minutes at 4°C. The ethanol was removed while leaving the RNA pellet undisturbed and 500 µL of 70% ethanol was then added. The solution was vortexed and centrifuged at 8000xg for 20 minutes at 4°C. The ethanol was removed, and the pellet was incubated at 37°C until the remaining ethanol evaporated. The pellet was then re-suspended in 20 µL of Nuclease-Free water (Ambion #4387936).

3.6 REVERSE TRANSCRIPTASE PCR (RT-PCR)

cDNA was prepared using the Superscript III First-Strand Synthesis kit (Invitrogen #18080051). A total of 1 µg of RNA was used to synthesize cDNA in a final volume of 10 µL. PCR reactions were performed using Phusion® High-Fidelity DNA polymerase (New England BioLabs #M0530S) and the following cycling parameters: denaturation at 98°C for 1 min, then 30 cycles of [98°C for 10 s, 60°C for 30 s, and 72°C for 30 s/kb], with a final extension step at 72°C for 10 minutes. For DrLITR 15.1, the annealing temperature was 66°C and rest of the parameters were identical. Elongation factor 1α (EF1α) was used as the loading control to ensure the integrity of cDNA. The primers used for RT-PCR are displayed in Table 3.1.

3.7 QUANTITATIVE PCR (qPCR)

3.7.1 Primer validation

All primers used in the qPCR experiments were validated using a serial dilution series to determine efficiencies. Primer validation and testing amplification efficiency are important to ensure that only the target gene is being amplified without any non-specific amplification occurring. Additionally, the correct concentration of primers must be added to ensure that

secondary structures like primer-dimers are not being created. The endogenous control used for all qPCR assays was β-actin because the threshold cycle (C_t) values between samples differed only by ±2. This indicates that the endogenous control was fairly unaffected by any treatments that the fish were subjected to. The C_t value represents the number of cycles that are required for the fluorescent molecules in SYBR™ Green to cross the threshold levels. SYBR™ Green is a dye that does not fluoresce when it is free-floating in solution. At the beginning of the qPCR reaction, extremely low fluorescence is detected (i.e. background levels). As the reaction proceeds, the fluorescent signal exceeds the background signal as the target sequence is amplified resulting in the determination of the C_t value. These values are mainly dependent on the amount of target in the PCR reaction. For example, if there is a lot of target sequence available then the C_t value will be low because the product will be detected faster and the fluorescent signal will pass the threshold quickly. The primers used for qPCR are displayed in Table 3.2 as well as their final working concentrations and their efficiencies. The slope of log of input vs C_t must be between -0.1 and 0.1. This is to ensure that primers are amplifying the targets at approximately the same efficiency as the reference amplification. Furthermore, melt curves of all primers were analyzed to confirm that non-specific amplification was not occurring.

3.7.2 qPCR PARAMETERS

QuantStudio 6 Flex Real-Time PCR system was used to run all qPCR assays (ABI Applied Biosystems). All the analysis was also done on the manufacturer's provided software. The PCR reaction was set up in a total volume of 10 µL containing 5 µL of SYBR Green Master Mix (MBSU, University of Alberta), 2 µL of cDNA, 1 µL of primer mix and 2 µL of water. The cDNA

was diluted 1 in 10 for all qPCR assays. The cycling conditions were as follows: initial denaturation for 2 min at 95 °C, 40 cycles of [95 °C for 15 s followed by 60 °C for 1 min]. Each biological replicate was tested in triplicates.

3.8 SEQUENCING

All PCR products (qPCR and RT-PCR) were subject to sequencing to confirm the specificity of the gene of interest. PCR reactions were separated on a 0.8% TAE-agarose gel and visualized by staining with SYBR^{TM} safe DNA gel stain (Invitrogen #S33102). Amplified PCR products were then gel extracted (Qiagen Gel Extraction Kit #28704) and cloned into pJET1.2/blunt using the blunt-end ligation protocol (CloneJET PCR cloning kit, Thermo Scientific #K1231). These ligation reactions were transformed into DH5α cells on Luria-Bertani (LB) plates with 50 µg/mL ampicillin and incubated at 37°C overnight. Colony PCR was performed the next day to check for successfully transformed colonies using *Taq* DNA polymerase (Invitogen #10342053). The PCR conditions were as follows: denaturation at 95°C for 5 minutes, then 25 cycles of [95°C for 30 s, 60°C for 30 s, and 72°C for 1 min/kb], with a final extension step at 72°C for 5 minutes. All colony PCR reactions were performed using pJET 1.2 forward and reverse primers (Table 3.1). The reactions were visualized, as mentioned above. Positive colonies were grown in LB medium with 50 µg/mL ampicillin and incubated at 37°C overnight. The plasmids were isolated using the QIAprep® Spin Miniprep Kit (Qiagen #27104) and sequenced at Molecular Biology Service Unit (MBSU) at the University of Alberta.

3.9 STATISTICS

All adult zebrafish data was log-transformed (log base 2) to normalize the distribution. A student's t-test was performed on the transformed data to analyze the differences among PBS

and zymosan treatments. A one-way ANOVA was used to analyze the embryonic development data followed by a post-hoc Tukey analysis. In both cases, P-values < 0.05 were considered significant. All statistics were performed on Prism 6 (GraphPad).

Table 3.1. RT-PCR primers used in this thesis. Primers were ordered from Integrated DNA Technologies (IDT). [a]Primers were obtained from Casadei et al. (2011). [b]Primers were obtained from ThermoScientific (#K1231)

Primer Name	Primer Sequence 5' to 3'
DrLITR 1.1 Fwd	AGGTTTCTCATATTGTCAGCAGTA
DrLITR 1.1 Rvrs	AGAGTTAACATGGCGTGTGTAT
DrLITR 1.2 Fwd	AGAGGAAGCAGGAAGTGAAAC
DrLITR 1.2 Rvrs	CACCTGATGGGAGTGAAGAAA
DrLITR 15.1 Fwd	CAGCACTGTGGAAGACGTTT
DrLITR 15.1 Rvrs	GTCATTGAGGCACCTGAGAATA
DrLITR 23.1 Fwd	CAGAACCACCTCAACCGAAA
DrLITR 23.1 Rvrs	GCATTCTCAGCAATGGGAAATC
EF1α Fwd[a]	CTGTACCTGTGGGTCGTGTGGAGACTG
EF1α Rvrs[a]	CAGCCTTCTGTGCAGACTTTGTGACC
pJET 1.2 Fwd[b]	CGACTCACTATAGGGAGAGCGGC
pJET 1.2 Rvrs[b]	AAGAACATCGATTTTCCATGGCAG

Table 3.2. qPCR primers used in this thesis. Primers were ordered from Integrated DNA Technologies (IDT). [a,e]Primers obtained from Guo et al. (2017). [b]Primers obtained from Fleisch et al. (2013) [c]Primers obtained from Wang, Zhang, & Wang (2008) . [d]Primers obtained from Zhang et al. (2012). [f,g]Primers obtained from Oyarbide, Rainieri, & Pardo (2012).

Primer Name	Primer Sequence 5' to 3'	Slope of log of input vs C_t values	Final Concentration (nM)
IL1β Fwd[a]	TTCCCCAAGTGCTGCTTATT	0.07	300
IL1β Rvrs[a]	AAGTTAAAACCGCTGTGGTCA		
β-actin Fwd[b]	CGGACAGGTCATCACCATTG	N/A	300
β-actin Rvrs[b]	GATGTCGACGTCACACTTCA		
C6 Fwd[c]	ATGACGCTGGCAAGGAAACT	0.04	500
C6 Rvrs[c]	TGTCTGAACCGCAGGGCTC		
IL8 Fwd[d]	GTCGCTGCATTGAAACAGAA	0.09	300
IL8 Rvrs[d]	CTTAACCCATGGAGCAGAGG		
TNFα Fwd[e]	ACAGACTGGGCACAGACACG	0.07	50
TNFα Rvrs[e]	AACATTTTCCTCACTTTCGTTCA		
TLR22 Fwd[f]	CCAGCTCTCGCCGTACCA	0.09	100
TLR22 Rvrs[f]	TTGGGCCAGCGGATGT		
MPO Fwd[g]	CAATGGCCCGCATAATCTG	0.05	500
MPO Rvrs[g]	GCGAAAAGGATCTCTGGGAACT		
DrLITR 1.1 Fwd	CCTTGACCCTAAGTCTAGATGC	0.07	100
DrLITR 1.1 Rvrs	TGCAGAATCCTCATCTTCAGG		
DrLITR 1.2 Fwd	TGCTCTGAGTACGACACACTA	0.09	300
DrLITR 1.2 Rvrs	TGTGGAACCATCATCTTCACG		
DrLITR 15.1 Fwd	AGAGCCTACCGACACTGACTAC	0.05	300
DrLITR 15.1 Rvrs	CGTTTCCACCTGCTCATCTC		
DrLITR 23.1 Fwd	CTCACTAATGGTGCTTCAGTCA	0.09	300
DrLITR 23.1 Rvrs	GATGTCAAGCCATTTGTTCCTG		

CHAPTER IV

CLONING, SEQUENCING AND CHARACTERIZATION OF CANDIDATE ZEBRAFISH LITR-TYPES

4.1 INTRODUCTION

Teleost leukocyte immune-type receptors (LITRs) were originally discovered in channel catfish over a decade ago (Stafford *et al.*, 2006). They are a multi gene family that encode both stimulatory and inhibitory receptor-types. Functional characterization of IpLITR-types *in vitro* using transfected cells has shown that these receptors can mediate various cellular effector functions in immune and non-immune mammalian cell lines (Cortes *et al.*, 2012; Montgomery *et al.*, 2012; Lillico *et al.*, 2015). Recently, IpLITRs have also been implicated in catfish anti-viral responses (Taylor *et al.*, 2016). Specifically, channel catfish were immunized with channel catfish virus (CCV)-infected MHC-matched clonal T cells (G14D-CCV) and PBLs were isolated after immunization. Then using the IpLITR-specific mAb CC41, cytotoxic T cells (CTLs) that lyse virus-infected cells were identified. CC41 recognizes an isoform of IpLITR 1.1a that is up-regulated on anti-viral CTLs. The CC41+ CTLs are able to kill viral-infected targets. Conversely, when these CTLs are pre-incubated with CC41 mAb, the lysis of virus-infected cells decreased by ~40%. This suggests that LITRs may have an important role in recognizing viral targets and mediating cytotoxicity. In general, LITR transcripts have only been characterized in channel catfish but it is theorized that other teleost species may also possess them.

When LITRs were discovered, there was no catfish genome available. This limited the scope of LITR research as the chromosomes these receptors were found on was not known and the intron/exon organization was also unknown. More importantly, the full scope of catfish LITRs could not be easily identified and characterized. For instance, allogenic stimulation of

catfish PBLs resulted in the generation of many LITR-like transcripts (Stafford *et al.*, 2007). However, it was unknown if these were splice variants or if they were different genes encoding similar Ig domains. If the genome was available, this question could have easily been answered by tracing their positions on chromosomes. Furthermore, it is unknown if all catfish LITRs encode the same Ig domains or if there is variability among LITRs within and between different chromosomes. Apart from the genome limitation, LITR characterization has only be limited to adult fish since studying development in catfish is extremely time-consuming as they take years to mature. Therefore, attempts to identify IpLITR in the zebrafish model, which has a sequenced genome and is a fish with rapid developmental stages, were made as described below.

Stafford et al. (2006) previously mined the zebrafish genome to identify putative LITR-types and reported the partial sequences of five LITR-like genes in the genome but no expression studies were performed. Montgomery et al. (2011) also performed BLASTp searches to identify putative LITR-types in other teleost species. The results indicated that potential LITR-types are present in zebrafish (*Danio rerio*), Atlantic salmon (*Salmo salar*), green pufferfish (*Tetradon nigrovidis*), halibut (*Paralichthys olivaceus*) and tiger pufferfish (*Takifugu rubripes*). Additionally, it has been estimated that zebrafish may contain up to at least 137 LITR Ig domains and that zebrafish LITR-types share 33-58% amino acid identity with catfish LITRs (reviewed by Rodríguez-Nunez *et al.*, 2014).

This chapter will further focus on the identification and characterization of zebrafish LITR-types. This is the first time LITRs are being characterized in a different teleost species. The zebrafish system can be used to investigate new avenues of LITR research that are not possible

in the catfish system. There is currently no information on intron/exon arrangement of LITRs, chromosome locations, sequence variation among LITR Ig domains, the presence/absence of isoforms and the regulation of LITRs during ontogeny. All of these questions can be answered using zebrafish as a model organism, which is why I decided to pursue LITR research in this teleost species. Since its been shown, via similarity searchers, that potential IpLITR-type transcripts exist in zebrafish, I predict that there will be protein sequence differences between the reference genome and the expressed cDNA. This is because the fish that was used to create the reference genome and the fish I used were different individuals. Additionally, LITR display polymorphisms as 16 catfish siblings show different DNA banding patterns using a single D1 probe when visualized via Southern blot (Stafford *et al.*, 2006). Therefore, there might be considerable differences between the reference genome and the expressed cDNA.

As mentioned in the previous chapter, I used BLASTp searches in the Ensembl database to identify potential LITR-types using the original three IpLITR prototypes as seed sequences (i.e. IpLITR1, IpLITR2 and IpLITR3). My specific objectives were to identify LITR-types using similarity searches and choose candidate zebrafish LITR-types that had unique CYT regions when compared to one another. Lastly, I wanted to perform additional similarity searches to investigate if zebrafish LITR-types are distantly related to the same mammalian proteins as IpLITRs or if there are any variations due to the difference in the type of teleost species used. To remain consistent with the previous catfish naming conventions, candidate LITR-types in zebrafish will be referred to as *Danio rerio* leukocyte immune-type receptors (DrLITRs).

Here I will report on the cloning and sequencing of four prototype DrLITRs: DrLITR 1.1, DrLITR 1.2, DrLITR 15.1 and DrLITR 23.1. These DrLITRs were expressed throughout ontogeny

and adulthood and by using the reference genome, I created intron/exon schematics for these receptors and annotated their structural features (i.e. Ig domain, TM and CYT region compositions). All Ig domains of the DrLITR-types described are at least ~30% similar to one another. The PSI-BLAST tables support the relatedness of DrLITRs and IpLITRs. Additionally, DrLITRs are distantly related to mammalian FcRLs, FcRs, sialic acid-binding Ig-like lectins (SIGLECs) and carcinoembryonic antigen-related cell adhesion molecules (CEACAMs).

4.2 RESULTS

4.2.1 Discovery of candidate DrLITRs

IpLITR1, IpLITR2 and IpLITR3 protein sequences were used to seed BLASTp searches against the zebrafish genome on Ensembl. I compiled a list of all the results and analyzed the sequences more closely. I had to ensure that these proteins contained Ig domains, a TM region and a short or long CYT region. If all of these requirements were met, I considered them putative IpLITR-types. I analyzed the CYT regions closely as I wanted to pick receptors that had unique CYT regions when compared to one another. I decided to pursue four candidate genes (i.e. DrLITR 1.1, DrLITR 1.2, DrLITR 15.1 and DrLITR 23.1) as they demonstrated unique signalling capacities as dictated by their cytoplasmic tails. I chose a new naming convention for these LITRs when compared to IpLITRs because I wanted their name to convey more information about their location in the genome. I decided to incorporate their chromosome location as well as the order in which they were found. For instance, DrLITR 15.1 is found on chromosome 15 and it is the first one to be found on this chromosome. Similarly, DrLITR 1.2 is found on chromosome 1 and it is the second one to be found on this chromosome. In general, the number before the decimal point is the chromosome location and the number after is the order

in which it was found. Based on the precited cDNAs, I created intron-exon structures for all four of the putative DrLITRs. DrLITR 1.1 and DrLITR 1.2 both contain three Ig domains, a TM region and a CYT region with both ITAM and ITIM motifs (Figures 4.1A and 4.2A; top intron/exon schematic). Additionally, DrLITR 1.2 contains a signal peptide, while DrLITR 1.1 is a partial sequence due to the absence of a start codon. DrLITR 15.1 contains three Ig domains, a TM segment and CYT region that includes two ITIMs and one ITSM (Figure 4.3A; top intron/exon schematic). DrLITR 23.1 contains four Ig domains, a positively charged TM region (i.e. due to the presence of lysine, K) as well as a short CYT region (Figure 4.4A; top intron/exon schematic). The structural composition of the receptors mentioned above is purely based on the predicted genome.

4.2.2 Cloning and sequencing of DrLITRs

I designed primers in the untranslated regions (UTR) of the predicted sequences of DrLITR 1.1, DrLITR 1.2, DrLITR 15.1 and DrLITR 23.1 to capture the entire coding regions of these receptors. I performed RT-PCR to visualize the expression of these transcripts. The results suggest that DrLITR 1.1, DrLITR 1.2, DrLITR 15.1 and DrLITR 23.1 are expressed ubiquitously throughout ontogeny and adulthood (Figures 4.1C, 4.2C, 4.3C, 4.4C). I cloned and sequenced these bands to acquire expressed cDNA transcripts and compared them to the reference genome. The prediction for DrLITR 1.2 was accurate as the cDNA transcript had >95% amino acid identity when compared to the predicted cDNA. Additionally, the structural composition of the receptor correctly matches the predicted structure (Figures 4.2A; bottom exon schematic, 4.2B). For example, DrLITR 1.2 contains a signal peptide, three Ig domains, a TM region and an ITAM ($Y_{356\text{-}368}$) and ITIM (Y_{391}) in its CYT region (Figure 4.5). DrLITR 1.1 was identified correctly as

a partial sequence and it does match the predicted genome perfectly with 100% amino acid identity. DrLITR 1.1 also contains three Ig domains, a TM region and an ITAM ($Y_{333-345}$) and ITIM (Y_{368}) in its CYT region (Figures 4.1A and B; 4.5).

DrLITR 15.1 and DrLITR 23.1 were annotated incorrectly by the Ensembl database. DrLITR 15.1 contains a signal peptide, four Ig domains, a TM region and a CYT region with two ITIMs and one ITSM (Figure 4.3A; bottom two intron/exon arrangements, 4.3B, 4.5). DrLITR 23.1 was annotated as a complete sequence but according to the cDNA, the sequence on Ensembl is a partial sequence. DrLITR 23.1 contains six Ig domains, a positively charged (i.e. lysine, K) TM, and a short CYT region with no known tyrosine-based motifs (Figure 4.4A; bottom two intron/exon arrangements, 4.4B, 4.5). There is no signal peptide present and the start codon is also absent, which makes this an incomplete transcript. When compared to the predicted coding regions, the amino acid identity of the cDNA transcripts were 72% and 80% for DrLITR 15.1 and DrLITR 23.1, respectively.

4.2.3 *In silico* analyses of DrLITRs

After I acquired the cDNA sequences, I wanted to examine how similar the Ig domains were to each other in the four DrLITRs characterized. The results suggested that the Ig domains were more similar to one another within each receptor when compared to their relatedness between the different receptors (Table 4.1, Figure 4.6). All Ig domains were at least 32% similar to one another (Table 4.1). The D3 domains of DrLITR 1.1 and DrLITR 1.2 were highly similar with 79% amino acid similarity. Within DrLITR 15.1, the D3 and D4 domains displayed 73% amino acid similarity. Within DrLITR 23.1, D1 and D3 shared 73% amino acid similarity and the D2 and D4 domains shared 88% amino acid similarity. Additionally, D5 is most similar (i.e. >51%

amino acid similarity) to D1, D2, D3 and D4 domains. I also performed PSI-BLAST to find DrLITR-related proteins in catfish and mammals. In channel catfish, the top hits always included IpLITRs as well as CEACAMs, FcRLs and SIGLECs (Tables 4.2-4.5). In mammals, the same top hits were also seen with a few additions such as pregnancy specific glycoproteins (PSGs).

4.3 DISCUSSION

The discovery of LITRs and all subsequent functional characterizations has been restricted to the channel catfish. This is the first time LITRs are have been characterized in another teleost species. To date, all of the IpLITR functional characterization experiments performed in our lab have depended on heterologous expression systems. These results are valuable as they answered important questions about the functional potential of these receptors but the role of LITRs *in vivo* is still unknown. My results show that LITRs are expressed in zebrafish tissues throughout development and adulthood. More importantly, I have cloned and sequences LITR-types in a different fish species that can be used as a tool to further understand the role of LITRs in fish immunity.

The availability of the zebrafish genome offers huge advantages. I was able to design primers on an existing reference genome to investigate the presence of putative LITR-types during specific stages of the zebrafish lifecycle. As a result, I was able to monitor LITR expression and analyze the differences between predicted and cDNA transcripts. DrLITR 1.1 and DrLITR 1.2 were predicted correctly. DrLITR 15.1 and DrLITR 23.1 were predicted incorrectly by the online database as having three and four Ig domains, respectively. The cloned cDNA suggested more Ig domains are present than what was predicted. This was not surprising as LITRs display polymorphism as 16 catfish siblings showed different IpLITR banding patterns on a

Southern blot analysis (Stafford *et al.*, 2006). This indicated that, within a species, there are multiple forms of LITR-types encoded. It is unknown if these multiple forms are all encoded by the same gene or different genes as the genome was not available to make a conclusion. Nonetheless, using a single IpLITR D1 probe, there was considerable variability in the DNA banding patterns. Therefore, it is expected that there is variability between the reference genome and the expressed cDNA of zebrafish as they came from different individuals. Additionally, DrLITR 1.1 and DrLITR 23.1 are partial sequences as the start codon is missing. I designed primers where I thought the start codon would be using the reference genome, but I was unable to pick up any products. Nevertheless, I decided to still examine them further due to their unique CYT regions. DrLITR 15.1 contains two overlapping ITIMs and an ITSM in its tail. IpLITR 1.1b contains the same motifs but the ITSM overlaps an ITIM. DrLITR 23.1 has six Ig domains and a positively charged TM as well as a single tyrosine residue present in its short CYT region, although this tyrosine does not belong to any recognizable tyrosine-based signalling motifs. IpLITR3 also contains six Ig domains, a positively charged TM and is devoid of tyrosines in its CYT region. Therefore, the CYT regions of DrLITR 15.1 and DrLITR 23.1 are different than what some of the known IpLITRs possess so there is an added possibility that these unique CYT regions may signal using novel pathways that were not previously observed in IpLITRs.

The Ig domains of DrLITRs are more similar within receptors than they are to each other. The only exception is that the D3 domains of DrLITR 1.1 and DrLITR 1.2 are highly similar to each other. The D1-D5 domains of DrLITR 23.1 are related while D3 and D4 of DrLITR 15.1 are related. These results are contrary to what is seen in catfish (Stafford *et al.*, 2006). In IpLITRs, the three original receptor prototypes had high amino acid identity between membrane distal

Ig domains. The Ig domain variation increases between membrane proximal domains. This pattern is opposite in DrLITR 1.1 and DrLITR 1.2 as the membrane proximal domains are more similar whereas the membrane distal domains have very low similarity. Additionally, the DrLITRs on the same chromosome (i.e. DrLITR 1.1 and DrLITR 1.2) have higher amino acid identity with each other than to DrLITRs on different chromosomes. It is possible that DrLITRs clustered on the same chromosome may bind similar ligands, which can be an explanation to their high amino acid similarity. It is also possible these two genes may have undergone multiple chromosomal duplication events that have diversified them from other DrLITRs but they retained their sequence identity. Additionally, all DrLITRs may have undergone genome duplication events, which resulted in the creation of highly diversified immunoglobulin domains. This phenomenon is observed in another teleost immunoregulatory family (i.e. NITRs) and therefore, can be the potential reason behind the highly variable Ig domains of DrLITRs (Yoder, 2009).

In humans, FcRs are found on chromosome 1 and they all bind the Fc portion of antibodies to trigger various innate immune responses (Takai, 2005; Nimmerjahn and Ravetch, 2007). On the other hand, KIRs are encoded by chromosome 19 and are involved in recognizing MHC molecules on healthy host cells (Trowsdale, 2001; Carrillo-Bustamante *et al.* 2016). Conversely, it was estimated that zebrafish contain at least 137 LITR-like Ig domains (Rodríguez-Nunez *et al.*, 2014). Therefore, the uniqueness of the Ig domains within DrLITRs is not overly surprising. It seems that DrLITRs encode these unique Ig domains within individual receptors as well as across different receptors. It is possible that these unique domains bind different ligands which could be an explanation for their sequence differences, as seen in the cases of FcRs and

KIRs. In addition, the characterized DrLITRs have varying number of Ig domains. This can affect the way they are oriented on the cell surface resulting in conformational differences among various receptors. For example, murine paired immunoglobulin receptor B (PirB) contains 6 Ig-like domains and adopts a "zig-zag" conformation (Vlieg, Huizinga and Janssen, 2019). Additionally, this receptor also shows flexibility and can display three more orientations between its D1 and D2 domains. It is suggested that receptors may show conformational flexibility to accommodate ligand binding. To conclude, extracellular portions have multiple functions and the high variability of Ig domains among DrLITRs may be an attribute for their potential diverse functions and variable roles in immunity.

Apart from the variability in the Ig domains, DrLITRs also contain unique CYT regions. DrLITR 1.1 and 1.2 both contain an ITAM and an ITIM in their CYT regions. For instance, FcRL5, in mice, contains an ITIM and an ITAM-like sequence in its CYT region (Davis *et al.*, 2001). The general consensus for an ITAM is D/ExxYxxL/I(x_{6-8})YxxL/I (Reth, 1989). FcRL5 is considered to have an ITAM-like motif because instead of an aliphatic residue at the second Y+3 position, there is a glutamic acid substitution (Davis *et al.*, 2001). In mice B1 cells, it was shown that this CYT region can mediate both stimulatory and inhibitory functions depending on the SHP-1 levels in its environment (Zhu *et al.*, 2013). If SHP-1 levels are low, the ITAM-like sequence recruits Lyn (Src-kinase family member) and activates calcium mobilization. However, if SHP-1 levels are high, B-cell activation is inhibited. On the other hand, DrLITR 15.1 contains two overlapping ITIMs and one ITSM. The same motifs are also seen in mice leukocyte mono-immunoglobulin-like receptor 3 (LMIR3; also called CD300f, an IgSF member) (Kumagai *et al.*, 2003). It has been shown that LMIR3 binds ceramide, a lipid, *in vivo* and inhibits mast cell

activation via ITIM- and ITSM-dependent pathways (Izawa *et al.*, 2012). Additionally, IpLITR 1.1b also contains two ITIMs and one ITSM and it has been shown to be functionally versatile in its signalling capacities (Montgomery *et al.*, 2012; Cortes *et al.*, 2014; Lillico *et al.*, 2015). Alternatively, DrLITR 23.1 contains a short CYT region with no tyrosine-based motifs but it does contain a positively-charged TM region. This receptor has the potential to interact with various adaptor molecules identified in zebrafish such as Dap10, Dap12, FcRγ, FcRγ-like, Cd3ζ and Cd3ζ-like, to stimulate immune responses (Yoder *et al.*, 2007). IpLITR 2.6b also contains a positively charged TM region and it was shown to bind negatively charged ITAM-containing IpFcRγ, IpFcRγ-like(L) and IpCD3ζ-L adaptor proteins (Mewes *et al.*, 2009). IpLITR 2.6b/IpFcRγ-L chimera was able to stimulate degranulation, phagocytosis and cytokine secretion (Cortes *et al.*, 2012, 2014). Altogether, DrLITR 1.1 and DrLITR 1.2 can be considered both putative stimulatory and inhibitory. DrLITR 15.1 is a putative inhibitory receptor while DrLITR 23.1 is a putative stimulatory receptor. It is tempting to distinguish receptors as either stimulatory or inhibitory based solely on their tyrosine-based motifs, but these motifs do show functional ambiguity. It has been shown for instance that FcαRI, in combination with its ITAM-containing adaptor protein FcRγ, can recruit both Syk and SHP-1 to its two tyrosines simultaneously (Pasquier *et al.*, 2005). When FcαRI continues its association with IgA complexes, it results in the activation of degranulation in RBL-2H3 cells. However, if there is no continued association, FcαRI inhibits degranulation.

ITSMs can also mediate both stimulatory and inhibitory responses (Schatzle *et al.*, 1999; Stepp *et al.*, 1999). In NK cells, a long variant of 2B4 (2B4L), a murine receptor, inhibited tumor lysis while the short variant, 2B4S, stimulated the process. In our lab, we have shown that ITIM-

containing IpLITR 1.1b can stimulate phagocytosis in RBL-2H3 cells (Cortes *et al.*, 2014; Lillico *et al.*, 2015). My results show that tyrosine-based motifs are present in the CYT regions of DrLITR 1.1, DrLITR 1.2 and DrLITR 15.1. In mammals, these tyrosine-based motifs show functional ambiguity dependent on the context of their environment. In IpLITRs, ITIM-containing IpLITR 1.1b have also shown functional versatility in their modulation of immune responses. Therefore, it is possible that DrLITRs may also display functional plasticity, but further functional investigation is required to make any concrete conclusions.

Similarity searches indicated common related proteins between DrLITRs and channel catfish and mammals. SIGLECs, FcRLs, and CEACAMs were among the top hits of mammals and channel catfish. Stafford et al. (2006) found that IpLITRs are closely related to FcRLs and LRC-encoded molecules (i.e. KIRs and LILRs). DrLITR 1.1, DrLITR 1.2, DrLITR 15.1 and DrLITR 23.1 are also related to FcRLs but they are not related to KIRs and LILRs. However, they are related to other IgSF receptor families in the LRC-encoded complex of mammals: SIGLECs, CEACAMs and PSGs (Varki and Angata, 2006; Crocker, Paulson and Varki, 2007; Barrow and Trowsdale, 2008). SIGLECs, such as CD22 and sialoadhesin, are a family of receptors that have shown to be rapidly diversifying due to gene duplications and exon shuffling (Angata *et al.*, 2004; Crocker, Paulson and Varki, 2007). They bind sialic acid containing proteins (Crocker, Paulson and Varki, 2007; Bornhöfft *et al.*, 2018). Sialoadhesin, also known as, SIGLEC-1, is thought to be responsible for cell-to-cell interactions as well as inducing phagocytosis of sialylated microbes (Chang and Nizet, 2014). CD22, also known as, SIGLEC-2, is predominantly expressed on B-cells and it is an inhibitory receptor that downregulates B-cell signalling for various effector functions (Crocker, Paulson and Varki, 2007; Clark and Giltiay, 2018). CEACAMs are involved in cell-to-cell adhesion

while PSGs are found in the maternal bloodstream and are thought to modulate fetal immune responses (Gray-Owen and Blumberg, 2006; Moore and Dveksler, 2014). DrLITRs are distantly related to these various IgSF receptor families but this does not mean that DrLITRs also bind the same ligands. This distant relationship is more indicative of the notion that FcRLs and LRC-encoded molecules most probably evolved from a common ancestor supported by the fact that IpLITRs and DrLITRs encode Ig domains pertaining to both families into one receptor (Stafford *et al.*, 2006). In other words, it is possible that teleost innate immune receptors and mammalian innate immune receptors were once encoded on the same chromosome but have since evolved and diversified.

Similarity searches are quite informative when interpreting the expression patterns displayed by DrLITRs. All of the top hits are either receptors that mediate immune functions or proteins that are involved in cell adhesion. These findings are particularly interesting as my results show that DrLITRs are ubiquitously expressed during embryogenesis and adulthood. Development is a very dynamic process as there are a lot of biological changes happening simultaneously (Kimmel *et al.*, 1995). This includes a wide array of events ranging from cell division to phenotypical and morphological changes. This requires a great amount of intracellular communication that can be mediated by adhesion molecules. For instance, CEACAMs have been implicated in embryogenesis as a major mediator of cell migration that results in integration of cells into functional organs (Kuespert, Pils and Hauck, 2006). It Is possible that LITRs may take on a CEACAM-like role in embryogenesis. Regardless of age (adults vs. embryos), innate immune defense is key for the survival of the host. DrLITRs, based on their distant relationship to FcR and FcRLs, may be involved in modulating various immune responses

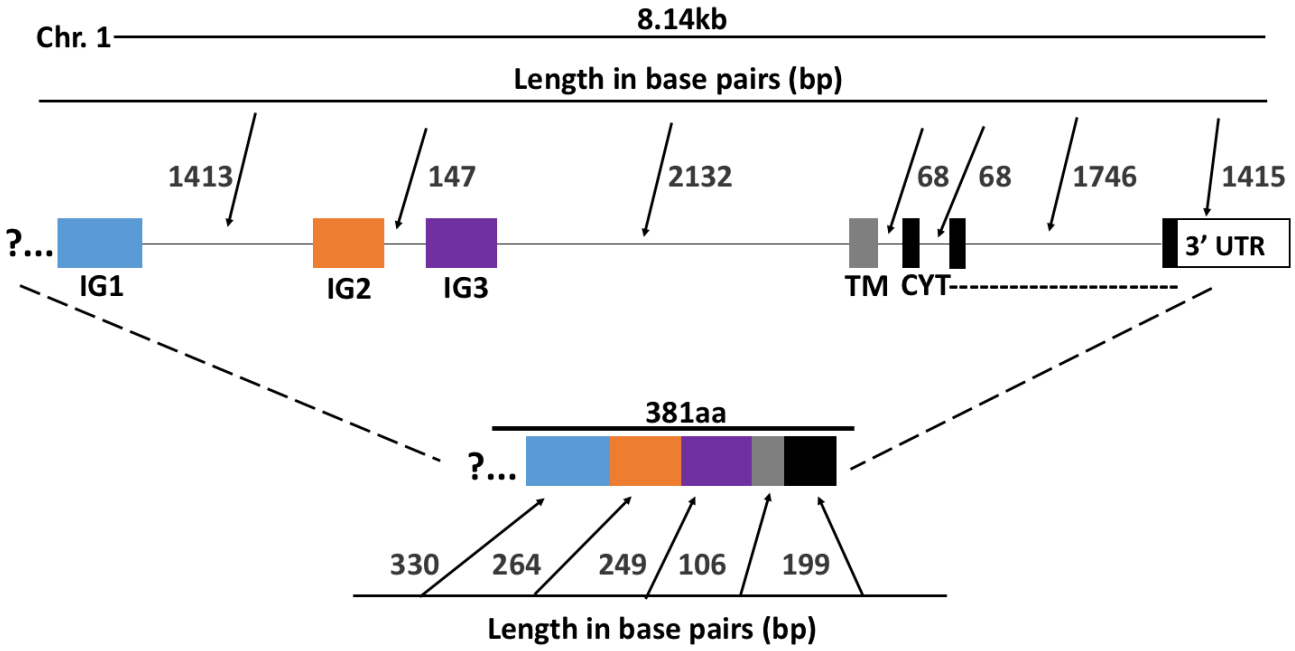
(Takai, 2005; Davis, 2007). It is important to note that these are all speculations solely based on the BLAST results. According to my data, I can only make the concrete conclusion that DrLITR 1.1, DrLITR 1.2, DrLITR 15.1 and DrLITR 23.1 are ubiquitously expressed.

DrLITRs show a different expression pattern than what is shown for the teleost-specific NITR family. Using RT-PCR, it has been shown that the majority of NITR-types seem to only be expressed in adult zebrafish tissues (Yoder *et al.*, 2010). This suggests that NITRs may not have any specific developmental roles. In contrast, DrLITRs are ubiquitously expressed regardless of the life stage. Admittedly, I have only characterized four DrLITR-types so I cannot generalize this result for all DrLITRs as there may be some that show NITR-like expression patterns. Nonetheless, the expression patterns of DrLITR 1.1, DrLITR 1.2, DrLITR 15.1 and DrLITR 23.1 do not follow the trend of most NITRs. This may mean that they are needed in some capacity in development or are involved in other key immune functions, but these are, once again, mere speculations. The only way to characterize their actual roles during ontogeny and adulthood is to perform functional studies *in vivo*.

To conclude, the availability of a reference genome has led to the discovery and characterization of four LITR-types in zebrafish. Additionally, they contain CYT regions that are unique between the four receptors but also unique when compared to some of the characterized catfish LITRs. BLAST results suggested that DrLITRs and IpLITRs are related proteins and similarity searches to the mammalian database has suggested distant relationships to FcRLs and the gene families found in the LRC complex. DrLITRs are ubiquitously expressed throughout embryogenesis and adulthood. RT-PCR expression analysis is a useful tool for examining the presence and absence of transcripts, but it is not a quantitative measure. The

quantitative levels are crucial to further elucidate the expression levels of the various DrLITRs during ontogeny and answer questions such as: are all DrLITRs expressed at the same levels? If not, do upregulated/downregulated transcripts mean that they are being differentially regulated as per the requirements of the embryonic stage? Do the embryos encode LITR transcripts natively or are they maternally sourced? I hope to provide insights to these questions, in detail, in the next chapter.

A.



B.

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> < Ig Domain 2

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> < Ig Domain 3

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> < CYT < ITAM >

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C.

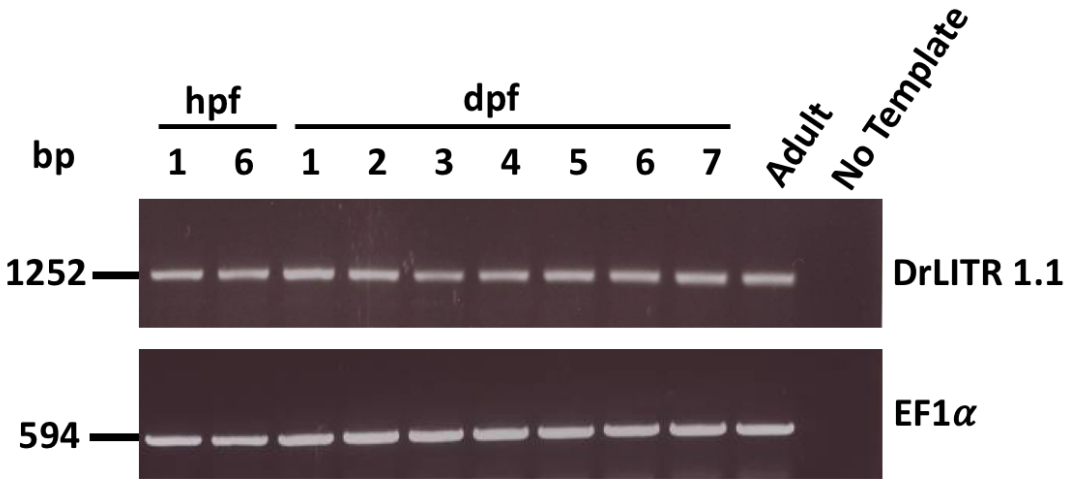
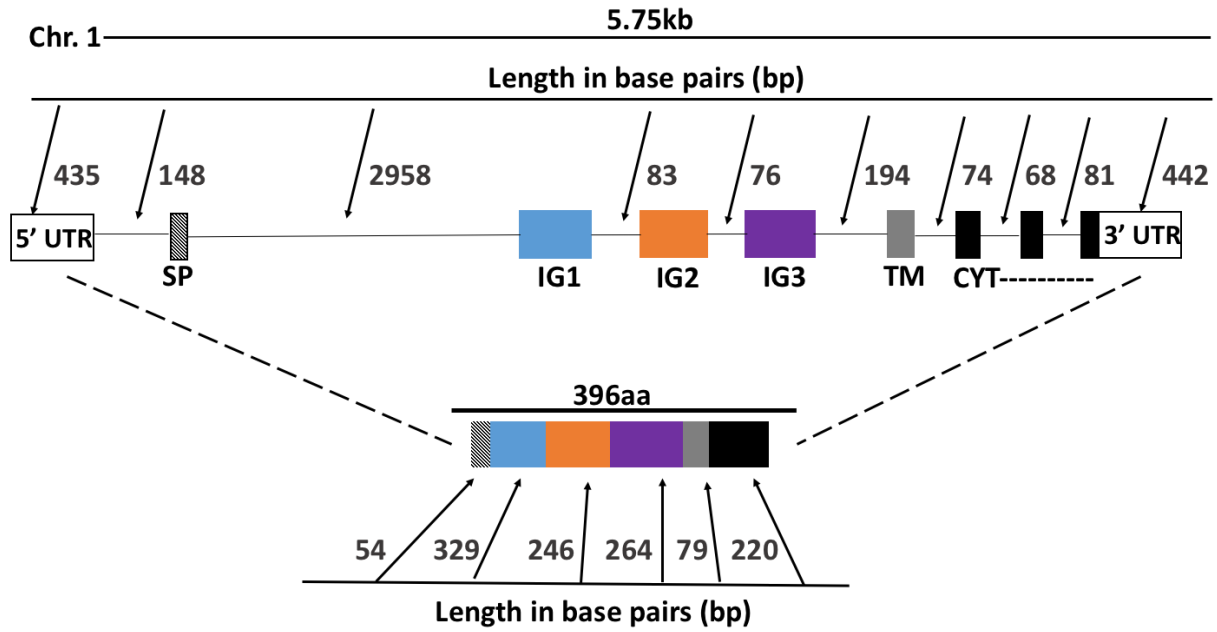


Figure 4.1. The gene schematic, structural features and expression profile of DrLITR 1.1. IpLITR1, IpLITR2 and IpLITR3 were used to seed BLASTp searches against the zebrafish genome in Ensembl (http://uswest.ensembl.org/index.html) to acquire the A) predicted intron/exon organization and predicted cDNA which I used to create primers to obtain the coding region which is 381 amino acids (aa). DrLITR 1.1 is a partial sequence, depicted by the question mark, as the 5' end is incomplete. The transcript is predicted to be 8.14 kilobases (kb) including the 3' untranslated (UTR) region and is located on chromosome 1. B) The protein sequence, based on the expressed cDNA, was acquired via ligation and transformation from a gel extraction of amplified C) RT-PCR products depicting the ubiquitously expressed receptor throughout ontogeny and adulthood. The time points chosen were 1 and 6 hours post fertilization (hpf), 1-7 days post fertilization (dpf), adult zebrafish viscera (spleen, intestines, pancreas, liver) and a no template lane. Elongation factor 1-alpha (EF1α) was used as a positive control to ensure that cDNA was intact and not degraded. The expected sizes of DrLITR 1.1 and EF1α are 1252 and 594 base pairs (bp), respectively. The immunoglobulin (IG) domains and the transmembrane region were annotated using Simple Modular Architecture Research Tool (SMART; http://smart.embl-heidelberg.de/). DrLITR 1.1 contains three immunoglobulin domains, a transmembrane (TM) region and an ITAM and an ITIM in its cytoplasmic (CYT) tail.

A.



B.

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> < Ig Domain 2

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> < Ig Domain 3

VSREDSGNYSCAVRDLNHLPSPALTLNIRYPPKSVSVSVSGSAVIESGDSVSLSCSSDSN

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C.

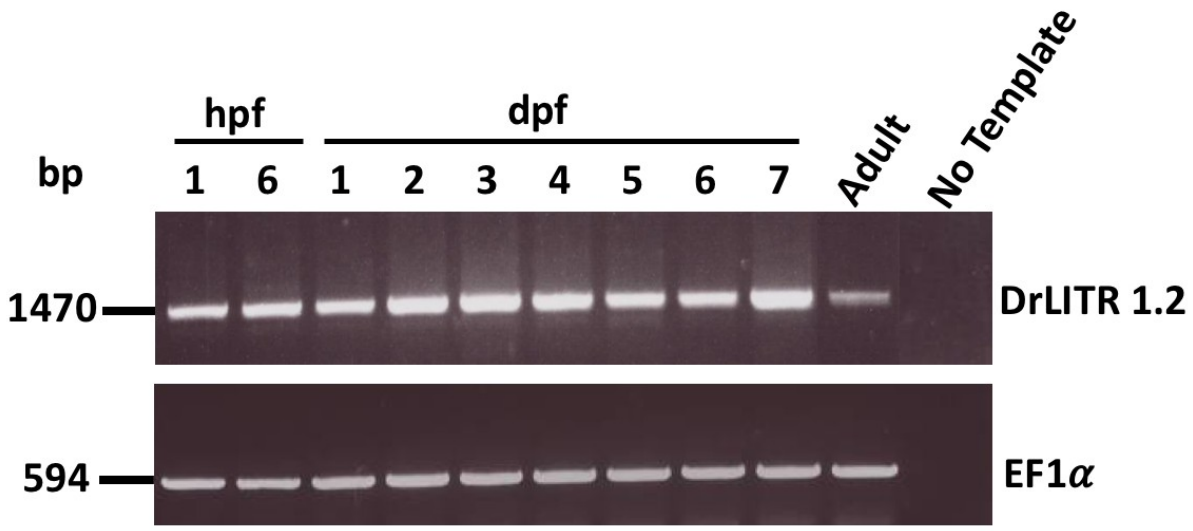
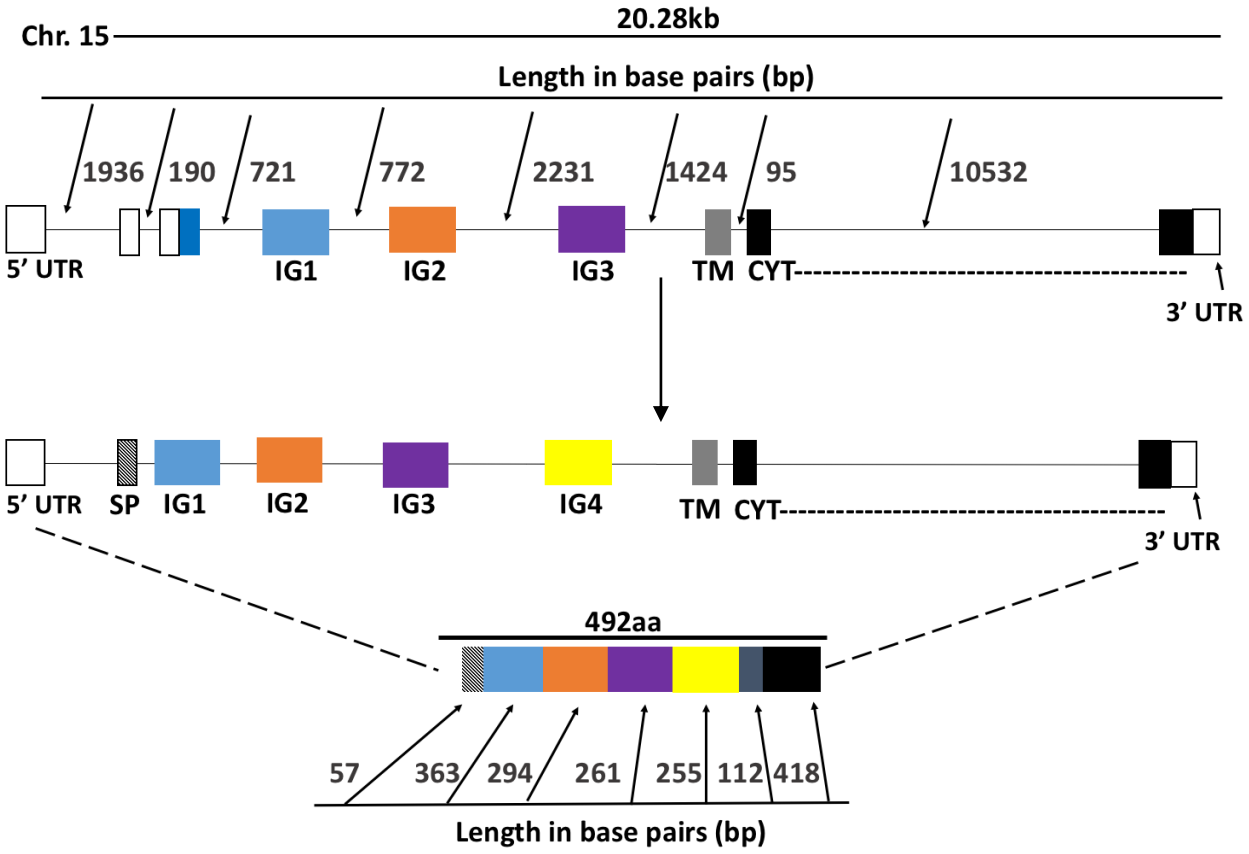


Figure 4.2. The gene schematic, structural features and expression profile of DrLITR 1.2. IpLITR1, IpLITR2 and IpLITR3 were used to seed BLASTp searches against the zebrafish genome in Ensembl (http://uswest.ensembl.org/index.html) to acquire the A) predicted intron/exon organization and predicted cDNA which I used to create primers to obtain the coding region which is 396 amino acids (aa) long. The transcript is predicted to be 5.75 kilobases (kb) including the untranslated (UTR) regions and is located on chromosome 1. B) The protein sequence, based on the expressed cDNA, was acquired via ligation and transformation from a gel extraction of amplified C) RT-PCR products depicting the ubiquitously expressed receptor throughout ontogeny and adulthood. The time points chosen were 1 and 6 hours post fertilization (hpf), 1-7 days post fertilization (dpf), adult zebrafish viscera (spleen, intestines, pancreas, liver) and a no template lane. Elongation factor 1-alpha (EF1α) was used as a positive control to ensure that cDNA was intact and not degraded. The expected sizes of DrLITR 1.2 and EF1α are 1470 and 594 base pairs (bp), respectively. The signal peptide (SP), immunoglobulin (IG) domains and the transmembrane (TM) region were annotated using Simple Modular Architecture Research Tool (SMART; http://smart.embl-heidelberg.de/). DrLITR 1.2 contains a signal peptide, three immunoglobulin domains, a transmembrane region and an ITAM and an ITIM in its cytoplasmic (CYT) tail.

A.



B.

< Signal peptide > < Ig Domain 1

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> < Ig Domain 2

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>

KLDNNKITQVPSEHVYYSNLTLIPKSDMHNGTVGCDARYGNKTISTKKTLVVIYVQALQI

< Ig Domain 3

VGKNTLMAGDTLNLTCTDRSHPPASNPVWRFNGNTDNLNTQTSAGTLMIANVGKEHAGIY

> < Ig Domain 4

VCEMTYMNKTLNASITINITDVQVEILGKNTLKEGDSLNLNCTIKNHSQALSDPVWSFNG

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PKEILINQTGGNLTIAKVGKKHAGIYVCEMTYMNKRLNASITINIITGVLDFLLSIPNIF

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> >

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C.

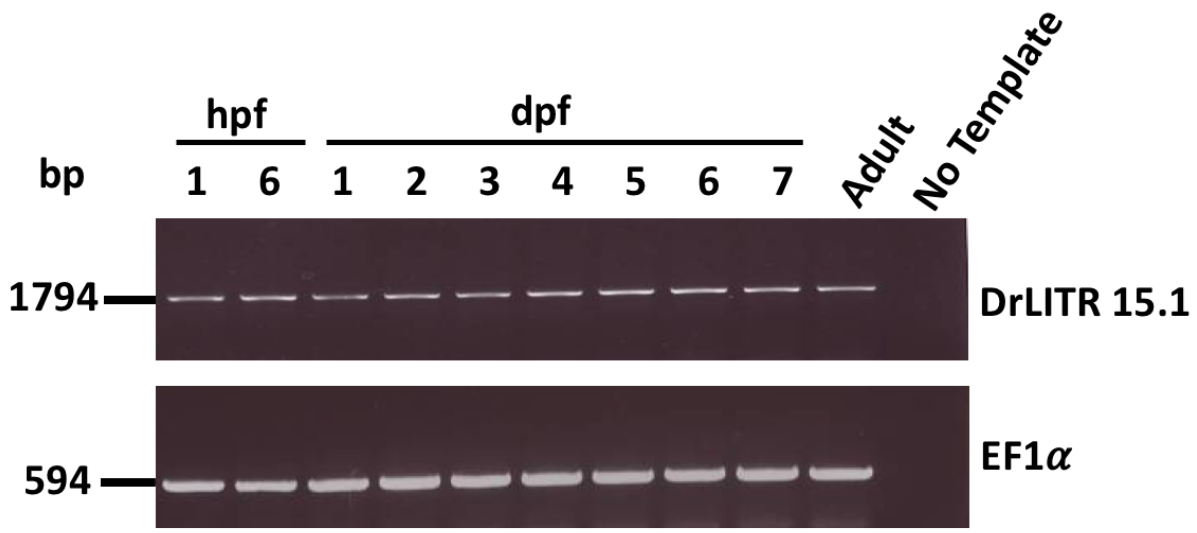
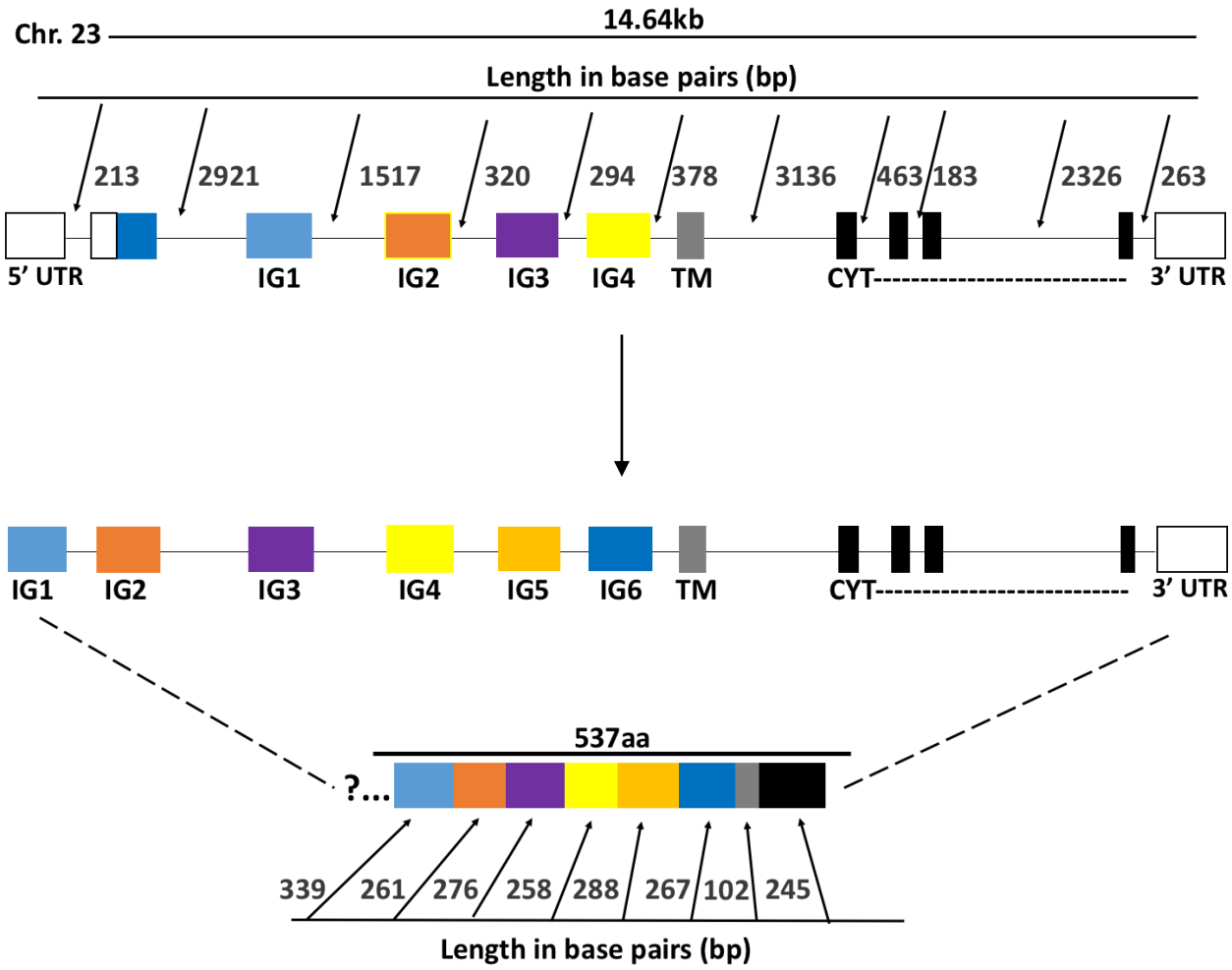


Figure 4.3. The gene schematic, structural features and expression profile of DrLITR 15.1. IpLITR1, IpLITR2 and IpLITR3 were used to seed BLASTp searches against the zebrafish genome in Ensembl (http://uswest.ensembl.org/index.html) to acquire the A) predicted intron/exon organization and predicted cDNA which was used to create primers to obtain the correct intron/exon organization. The coding region is 492 amino acids (aa) long. The transcript is predicted to be 20.28 kilobases (kb) including the untranslated (UTR) regions and is located on chromosome 15. B) The protein sequence, based on the expressed cDNA, was acquired via ligation and transformation from a gel extraction of amplified C) RT-PCR products depicting the ubiquitously expressed receptor throughout ontogeny and adulthood. The time points chosen were 1 and 6 hours post fertilization (hpf), 1-7 days post fertilization (dpf), adult zebrafish viscera (spleen, intestines, pancreas, liver) and a no template lane. Elongation factor 1-alpha (EF1α) was used as a positive control to ensure that cDNA was intact and not degraded. The expected sizes of DrLITR 15.1 and EF1α are 1794 and 594 base pairs (bp), respectively. The signal peptide (SP), immunoglobulin (IG) domains and the transmembrane (TM) region were annotated using Simple Modular Architecture Research Tool (SMART; http://smart.embl-heidelberg.de/). DrLITR 15.1 contains a signal peptide, four immunoglobulin domains, a transmembrane region and 2 overlapping ITIMs and an ITSM in its cytoplasmic (CYT) tail.

A.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Length in base pairs (bp)
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1517
320
294
378
3136
463
183
2326
263
5' UTR
IG1
IG2
IG3
IG4
TM
CYT----------
3' UTR
IG1
IG2
IG3
IG4
IG5
IG6
TM
CYT----------
3' UTR
537aa
?...
339
261
276
258
288
267
102
245
Length in base pairs (bp)

B.

```
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                                                  >          < Ig Domain 2
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                                                  >          < Ig Domain 4
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          >        < TM                                  * > < CYT
NGFVANNLILKRKILLAISGCLVCCILILILGCIALKFMRKPVEKKDPTENDLFFKMTDS

NNQTATPLKEYMDNTAIEIAECKEIEELLTNGASVTQEGDAIKDEPVDSPAAGTNGLTSF

   >
KGI
```

C.

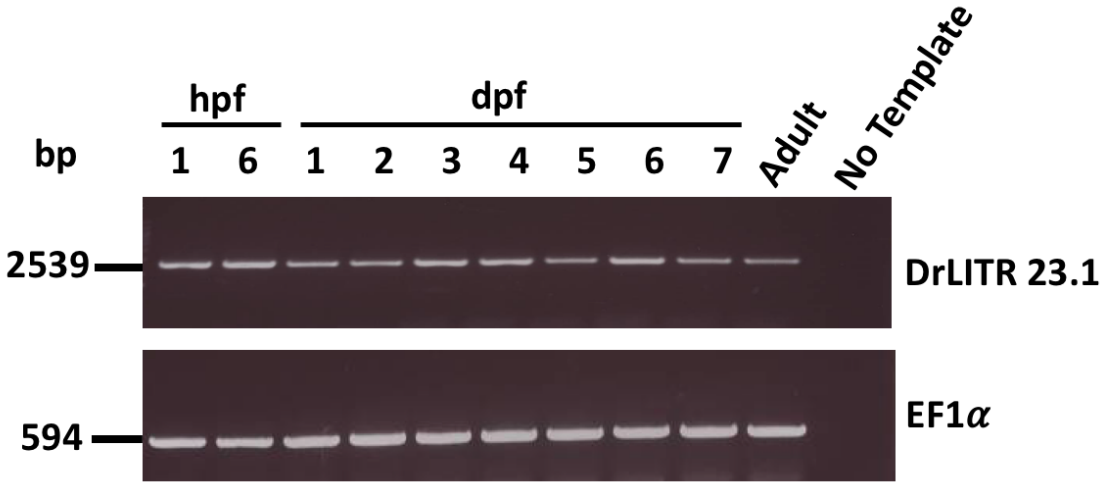


Figure 4.4. The gene schematic, structural features and expression profile of DrLITR 23.1. IpLITR1, IpLITR2 and IpLITR3 were used to seed BLASTp searches against the zebrafish genome in Ensembl (http://uswest.ensembl.org/index.html) to acquire the A) predicted intron/exon organization and predicted cDNA which was used to create primers to obtain the correct intron/exon organization. The coding region is 537 amino acids (aa) long. DrLITR 23.1 is a partial sequence, depicted by the question mark, as the 5' end is incomplete. The transcript is predicted to be 14.64 kilobases (kb) including the 3' untranslated (UTR) region and is located on chromosome 23. B) The protein sequence, based on the expressed cDNA, was acquired via ligation and transformation from a gel extraction of amplified C) RT-PCR products depicting the ubiquitously expressed receptor throughout ontogeny and adulthood. The time points chosen were 1 and 6 hours post fertilization (hpf), 1-7 days post fertilization (dpf), adult zebrafish viscera (spleen, intestines, pancreas, liver) and a no template lane. Elongation factor 1-alpha (EF1α) was used as a positive control to ensure that cDNA was intact and not degraded. The expected sizes of DrLITR 23.1 and EF1α are 2539 and 594 base pairs (bp), respectively. The immunoglobulin (IG) domains and the transmembrane region were annotated using Simple Modular Architecture Research Tool (SMART; http://smart.embl-heidelberg.de/). DrLITR 23.1 contains six immunoglobulin domains, a positively charged transmembrane (TM) region due to the presence of lysine, K, as indicated by the asterisk, and a short cytoplasmic tail with the presence of a tyrosine, Y, as indicated by the box, but does not contain any recognizable tyrosine-based motifs.

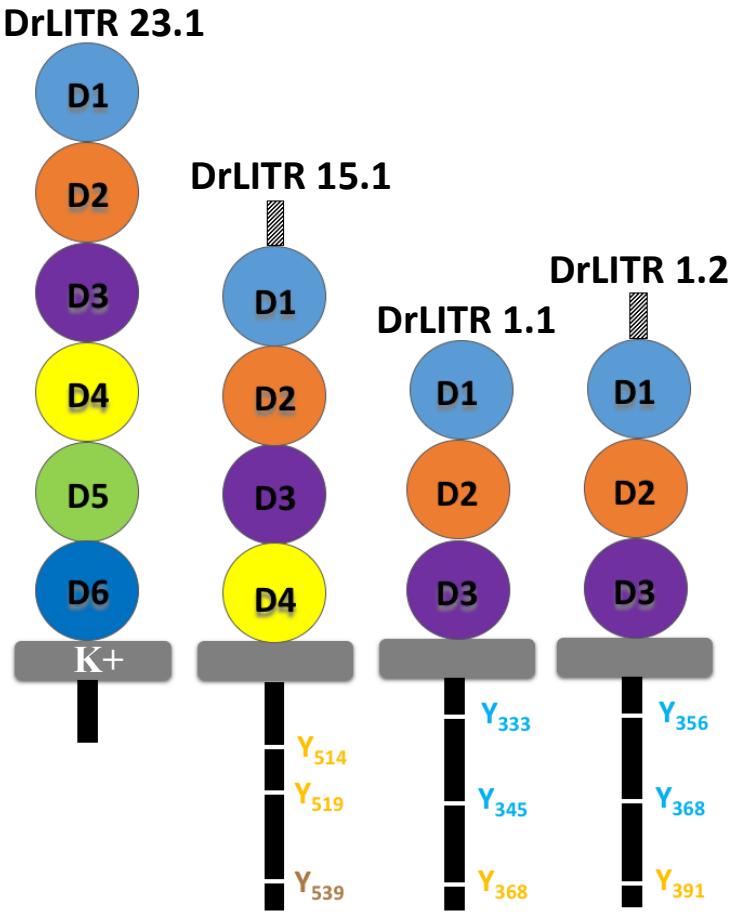


Figure 4.5. Schematic representation of DrLITR 1.1, DrLITR 1.2, DrLITR 15.1 and DrLITR 23.1. DrLITR receptor schematics were created based on the immunoglobulin (Ig) domains and the transmembrane (TM) segment predictions using the Simple Modular Architecture Research Tool (SMART; http://smart.embl-heidelberg.de/). The cytoplasmic (CYT) tails were labelled based on conserved tyrosine-based motifs. The colors used in this schematic match the colors of the intron-exon arrangements of Figures 4.1-4.4 and are not an indication of shared domain identities between the proteins. Additionally, the immunotyrosine-based activation motif (ITAM), immunotyrosine-based inhibitory motif (ITIM) and the immunotyrosine-based switch motif (ITSM) are labelled in blue, yellow and brown, respectively. DrLITR 23.1 has six immunoglobulin (Ig) domains labelled D1-D6, a positively charged (lysine, K+) TM segment and a CYT region devoid of any tyrosine-based motifs. DrLITR 1.1 and 1.2 have three Ig domains (i.e. D1-D3), a TM region and a CYT region comprising of an ITAM and an ITIM. DrLITR 15.1 has four Ig domains, a TM segment and a CYT region containing two ITIMs and one ITSM. In addition, DrLITR 1.2 and DrLITR 15.1 contain signal peptides, as indicated by the patterned bar.

Table 4.1. Amino acid similarity (%) table.

	1.1 D1	1.1 D2	1.1 D3	1.2 D1	1.2 D2	1.2 D3	15.1 D1	15.1 D2	15.1 D3	15.1 D4	23.1 D1	23.1 D2	23.1 D3	23.1 D4	23.1 D5	23.1 D6
1.1 D1		36	33	46	36	34	41	35	37	37	36	35	36	38	38	39
1.1 D2			38	40	36	34	39	38	37	42	32	39	36	39	36	33
1.1 D3				36	41	**79**	36	41	47	43	41	41	39	39	37	40
1.2 D1					38	34	37	39	38	40	37	32	38	33	37	36
1.2 D2						48	36	37	46	43	35	36	37	38	43	42
1.2 D3							35	44	49	45	43	45	41	46	43	39
15.1 D1								38	35	35	37	36	34	38	38	34
15.1 D2									40	35	40	37	37	36	39	34
15.1 D3										**73**	39	44	40	39	39	42
15.1 D4											40	41	42	42	43	42
23.1 D1												**55**	**73**	**51**	**51**	46
23.1 D2													**53**	**88**	44	44
23.1 D3														**51**	**51**	43
23.1 D4															46	42
23.1 D5																49
23.1 D6																

Individual immunoglobulin domains of DrLITR 1.1, DrLITR 1.1, DrLITR 1.2, DrLITR 15.1 and DrLITR 23.1 were aligned using Emboss Stretcher, a global pairwise alignment tool, (https://www.ebi.ac.uk/Tools/psa/emboss_stretcher/) to calculate percent (%) similarity between all Ig domains of DrLITRs. Similarities of >50% are bolded.

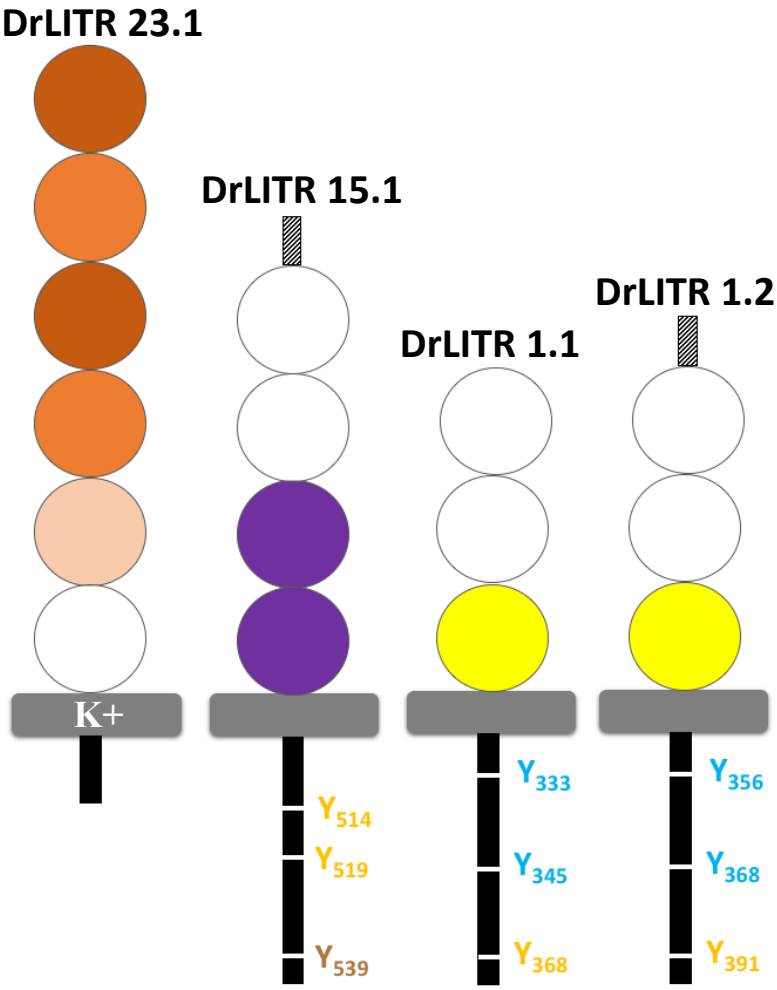


Figure 4.6. Schematic representation of DrLITR 1.1, DrLITR 1.2, DrLITR 15.1 and DrLITR 23.1 based on immunoglobulin domain similarity. DrLITR schematics were created based on the immunoglobulin (Ig) domains similarity depicted in Table 4.1. Domains with identical colors have amino acid similarities > 70% while the lighter shades indicate amino acid similarities of > 50%. The white colored Ig domains have < 50% similarity with all other domains. The immunoglobulin (Ig) domains and transmembrane (TM) segments were annotated using Simple Modular Architecture Research Tool (SMART; http://smart.embl-heidelberg.de/). Additionally, the immunotyrosine-based activation motif (ITAM), immunotyrosine-based inhibitory motif (ITIM) and the immunotyrosine-based switch motif (ITSM) are labelled in blue, yellow and brown, respectively. The signal peptide is represented by a patterned bar.

Table 4.2. Representative PSI-BLAST results of DrLITR 1.1.

DrLITR 1.1					
Top Matching Catfish Sequences	**E value**	**Max. Score**	**ID (%)**	**Pos. (%)**	**Coverage (%)**
B-Cell receptor CD22-like	7e-15	75.5	28.77	45	52
Sialoadhesin-like	2e-12	69.7	26.38	45	84
CEACAM1-like	1e-08	57.4	24.63	41	68
LITR TS32.17 L2.1a	3e-04	43.5	26.19	41	56
FcRL5	1e-03	42.0	25.48	45	61
Top Matching Mammal Sequences					
FcRL 2 (Monkey)	4e-14	80.9	25.62	42	76
Sialoadhesin (Manatee)	1e-12	77.4	24.48	45	68
VCAM-1 (Platypus)	2e-12	75.9	28.44	48	68
SIGLEC1 (Human)	1e-11	73.9	27.54	48	68
B-Cell receptor CD22 (Mole rat)	1e-11	73.6	31.84	47	68

Position Specific iterative (PSI) Basic Local Alignment Search Tool (BLAST) was used to search the nonredundant database of NCBI. The entire DrLITR 1.1 amino acid sequence was used to search the channel catfish and mammal databases to identify related proteins.

CD22: cluster of differentiation 22, *CEACAM:* carcinoembryonic antigen-related cell adhesion molecule, *LITR:* leukocyte immune-type receptor*, FcRL:* fragment crystallizable-like, *SIGLEC:* Sialic acid-binding Ig-like lectin, *VCAM:* Vascular cell adhesion protein.

Table 4.3. Representative PSI-BLAST results of DrLITR 1.2.

DrLITR 1.2					
Top Matching Catfish Sequences	**E value**	**Max. Score**	**ID (%)**	**Pos. (%)**	**Coverage (%)**
B-Cell receptor CD22-like	3e-20	89.4	35.52	53	42
sialoadhesin-like	9e-16	77.0	33.65	50	51
CD166 antigen	4e-13	72.0	26.86	47	43
LITR 1	4e-12	68.2	28.51	47	80
FcRL 5	3e-11	66.2	28.89	47	79
Top Matching Mammal Sequences					
CD22 (Human)	4e-23	108	28.85	48	66
SIGLEC 2 (Macaque)	5e-21	102	29.52	50	79
FcRL 1 (Marmot)	1e-17	91.3	27.39	46	72
PSG5 (Bonobo)	3e-16	86.3	28.43	44	70
CEACAM 6 (Grizzly Bear)	4e-14	80.5	28.10	42	69

Position Specific iterative (PSI) Basic Local Alignment Search Tool (BLAST) was used to search the nonredundant database of NCBI. The entire DrLITR 1.2 amino acid sequence was used to search the channel catfish and mammal databases to identify related proteins.

CD22: cluster of differentiation 22, *CD166:* cluster of differentiation 166, *CEACAM:* carcinoembryonic antigen-related cell adhesion molecule, *LITR:* leukocyte immune-type receptor*, FcRL:* fragment crystallizable-like, *SIGLEC:* Sialic acid-binding Ig-like lectin, *PSG:* pregnancy specific glycoprotein.

Table 4.4. Representative PSI-BLAST results of DrLITR 15.1.

DrLITR 15.1					
Top Matching Catfish Sequences	**E value**	**Max. Score**	**ID (%)**	**Pos. (%)**	**Coverage (%)**
B-Cell receptor CD22-like	2e-12	70.9	22.89	41	73
Sialoadhesin-like	4e-09	60.8	19.41	39	62
LITR TS32.15 L1.1a	9e-08	56.2	24.12	39	48
FcRL 5	1e-07	55.5	22.63	39	42
CEACAM 5-like	2e-06	52.0	22.84	39	43
Top Matching Mammal Sequences					
SIGLEC 5 (Human)	3e-18	95.5	22.12	39	87
Sialoadhesin (Meerkat)	3e-17	93.6	22.51	41	68
B-cell receptor CD22 (Platypus)	3e-14	83.6	24.42	40	61
CEACAM 1 (Monkey)	3e-10	70.1	23.94	41	76
FcγRIIB (Mouse)	6e-10	67.8	24.83	44	50

Position Specific iterative (PSI) Basic Local Alignment Search Tool (BLAST) was used to search the nonredundant database of NCBI. The entire DrLITR 15.1 amino acid sequence was used to search the channel catfish and mammal databases to identify related proteins.

CD22: cluster of differentiation 22, *CEACAM:* carcinoembryonic antigen-related cell adhesion molecule, *LITR:* leukocyte immune-type receptor*, FcRL:* fragment crystallizable-like, *SIGLEC:* Sialic acid-binding Ig-like lectin, *FcγRIIB:* fragment crystallizable gamma receptor IIB.

Table 4.5. Representative PSI-BLAST results of DrLITR 23.1.

DrLITR 23.1					
Top Matching Catfish Sequences	**E value**	**Max. Score**	**ID (%)**	**Pos. (%)**	**Coverage (%)**
FcRL5	2e-34	141	25.89	44	90
CEACAM 5	2e-31	131	23.68	43	90
LITR 1	6e-30	127	26.03	42	82
Sialoadhesin-like	1e-14	78.2	22.86	40	79
B-Cell receptor CD22-like	9e-13	72.4	25.74	45	63
Top Matching Mammal Sequences					
CEACAM 1 (Elephant)	2e-16	90.5	25.44	41	74
PSG1 (Monkey)	3e-14	81.3	27.85	42	43
FcRL5 (Naked Mole Rat)	4e-13	80.5	23.41	40	76
B-Cell receptor CD22 (Rat)	2e-09	68.9	22.42	39	89
Sialoadhesin (Degu)	9e-09	66.6	25.41	41	77

Position Specific iterative (PSI) Basic Local Alignment Search Tool (BLAST) was used to search the nonredundant database of NCBI. The entire DrLITR 23.1 amino acid sequence was used to search the channel catfish and mammal databases to identify related proteins.

CD22: cluster of differentiation 22, *CEACAM:* carcinoembryonic antigen-related cell adhesion molecule, *LITR:* leukocyte immune-type receptor*, FcRL:* fragment crystallizable-like, *SIGLEC:* Sialic acid-binding Ig-like lectin, *PSG:* pregnancy specific glycoprotein.

CHAPTER V

EXPRESSION ANALYSIS OF SELECT DrLITRs DURING EMBRYOGENESIS AND IN ADULTS USING A VISCERAL CAVITY-BASED INFLAMMATION MODEL

5.1 INTRODUCTION

The vertebrate innate immune system represents an organisms germline encoded and first line of immune defense throughout all life stages. Innate immunity is relatively non-specific as it does not require previous encounters with pathogens and can mount immune responses fairly quickly. In zebrafish, innate immune mediators, such as cytokines, are present at the very early stages of life and in adults the adaptive immune system is fully developed but the innate immune system continues to function as the first line of defense (Oyarbide, Rainieri and Pardo, 2012).

Various innate immune genes, such as IL1β, TNFα and C3a, in zebrafish viscera (i.e. liver, pancreas, spleen and intestines) are significantly upregulated when fish are injected intraperitoneally with an immunostimulant such as bacterial LPS (Gonçalves *et al.*, 2017). Additionally, exposure to zymosan (fungal carbohydrate) resulted in upregulation of cytokines in a zebrafish liver cell line (ZFL cell line) (Ruyra *et al.*, 2015). These results show that intraperitoneal injections can be used to measure the induction of the immune system (i.e. the inflammatory response) using the visceral cavity model in adult fish.

Innate immune responses, such as inflammation, are regulated by cell-surface expressed immunoregulatory receptors. In zebrafish, the expression of select innate immune receptors is documented. For example, researchers have identified key points during development where immunoregulatory genes such as TLRs and NITRs are detected (or not

detected) creating differential expression patterns (Van Der Sar *et al.*, 2006; Yoder *et al.*, 2010). TLRs regulate many immune processes such as recognizing pathogens, inducing production of proinflammatory molecules, and initiating signalling cascades to promote cell effector responses (Kumagai and Akira, 2010). NITRs are a fish-specific immunoregulatory receptor family consisting of stimulatory and inhibitory forms with the ability to regulate cell effector responses (Yoder, 2009). NITRs, as well as TLRs, have also been detected during various stages of development. For example, TLR2 is expressed as early as 1 hour post fertilization (hpf) while TLR1 is expressed after 4.3 hpf (Van der Sar et al., 2006). Similarly, NITR3 is ubiquitously expressed during all stages of development and adulthood while most other NITRs are only expressed in adulthood (Yoder *et al.,* 2010). In summary, TLRs are ubiquitously expressed throughout ontogeny and adulthood. Conversely, NITRs seem to be mostly expressed in adults, with the exception of NITR3. There is no developmental quantified gene expression data available for all of these receptors in fish but for other innate immune markers, such as cytokines, it has been shown that they are expressed at various levels during development (Oyarbide, Rainieri and Pardo, 2012). However, it is not known if these receptors are present solely for the purpose of the protection of the embryo or if they contribute meaningful functions during the development of the organism.

There is also no quantitative data available for LITR expression levels during development. However, in adult catfish, it is known that LITRs are expressed in the spleen, gill and kidney (Stafford *et al.*, 2006). They are also expressed in immune cells, such as macrophages, NK-like cells, T-cells and B-cells. It is difficult to perform developmental studies in catfish as they take years to mature while zebrafish take 3 months. This is one of the major

advantages for using zebrafish as their life cycle is relatively fast (Traver *et al.*, 2003). Therefore, LITR studies during development can be best achieved with zebrafish as a model organism. In the previous chapter, I showed that DrLITRs are ubiquitously expressed. Since there is no quantified gene expression for LITRs, I made predictions for DrLITR expression based on what is previously observed for other innate immune receptors, such as TLRs and NITRs. For instance, NITRs all belong to the same immunoregulatory receptor family yet display different expression profiles during development. TLRs also show the same trend of variable expression patterns. Therefore, I predict that DrLITR expression levels will also differ during ontogeny. This could be due to the idea that they have different functions during various stages of zebrafish life. For instance, all TLRs have different ligands and, therefore, are specific to various pathogenic challenges (i.e. bacterial, fugal and viral). Based on the differential expression patterns of TLRs, it is predicted that they may have different functions during development. In other words, the role of TLRs during immune responses is known but their role during ontogeny is still unknown. On the other hand, the role of DrLITRs during both ontogeny and immunity remains unknown. Based on the existing expression profiles of other innate immune receptors, I hypothesize that DrLITRs will follow a TLR-like and/or NITR-like trend, where the expression patterns will be variable throughout various stages of zebrafish life.

To resolve this hypothesis, an *in vivo* model needs to be established where these expression patterns can be studied. For LITRs, no such model has ever been developed. A major objective of my thesis was to establish zebrafish as an ontogeny and inflammation model to examine DrLITR expression patterns. Specifically, I developed a qPCR assay to quantify DrLITR expression during development and during zymosan-induced inflammation in adults. In the

previous chapter, I identified, cloned and sequenced four LITR-types in zebrafish. Additionally, I showed that DrLITR 1.1, DrLITR 1.2, DrLITR 15.1 and DrLITR 23.1 are ubiquitously expressed throughout development and adulthood using standard RT-PCR analysis. The main objective for this chapter was to establish zebrafish as a model to study the expression levels of DrLITRs during various life stages. Specifically, I aimed to analyze and interpret the transcript expression levels during various developmental stages. I also planned to analyze the transcript levels during zymosan-induced inflammation in adults using a viscera-based inflammation model. My data shows that during development, DrLITR 1.1, DrLITR 1.2 and DrLITR 23.1 have higher expression levels before 1 day post fertilization (dpf) while DrLITR 15.1 has higher expression levels after 2 dpf. Additionally, it seems DrLITRs are maternally sourced as DrLITR expression was detected in unfertilized eggs at comparable levels to fertilized embryos at 1 hpf. In adults, DrLITRs seem to not be directly involved in zymosan-induced inflammation. IL1β, a classical pro-inflammatory cytokine, was significantly upregulated at 3, 6, 8 and 12 hours post zymosan exposure. DrLITRs do not follow this pattern and the expression levels are variable among each receptor. This is the first time a qPCR assay has been used to monitor LITR expressing during development as well as during zymosan-induced inflammation. The role of LITRs remains unknown, however, their variable expression profiles suggests that each DrLITR may contribute to a different function during various stages of zebrafish life.

5.2 RESULTS

5.2.1 Examination of DrLITR expression during zebrafish development

Embryos were reared in egg water (60 mg/L Instant Ocean) and incubated at 28.5ºC. The experimental time points chosen for embryos were 0 hpf (unfertilized eggs), 1 hpf and 6

hpf as well as 1-7 days post fertilization (dpf). Each experiment contained 50 embryos per time point. All samples were normalized to the 1 dpf (i.e. reference sample) time point and β-actin (i.e. endogenous control) using the comparative C_t method. The reason I chose 1 dpf (i.e. 24 hpf) as the reference is because macrophages are detected at 1 dpf in embryos and, therefore, I chose to standardize my data to this time point as these immune cells are involved in all key cell effector responses (Herbomel, Thisse and Thisse, 1999). In all cases, p-values of < 0.05 were considered significant. The controls chosen for this experiment were C6, IL1β, IL8, MPO, TLR22 and TNFα. With the exception of IL8, all of these innate immune markers were expressed as early as 1 hpf (Figures 5.1-5.6). Specifically, IL8 was expressed after 1 dpf (Figure 5.3). DrLITR 1.1, DrLITR 1.2, DrLITR 15.1 and DrLITR 23.1 were expressed as early as 1 hpf (Figures 5.7-5.10). Overall, the general trends for C6, IL8, MPO, TLR22, TNFα and DrLITR 15.1 was higher expression levels after 1 dpf (Figures 5.1, 5.3, 5.4,5.5, 5.6, 5.9). The general trends for IL1β (p-value <0.001 at 6 hpf), DrLITR 1.1, DrLITR 1.2 and DrLITR 23.1 was higher expression levels at 1 hpf and 6 hpf (p-value <0.05) (Figures 5.2, 5.7, 5.8, 5.10). Additionally, DrLITR 23.1 had the highest expression at 1 hpf with ~300 fold increase relative to 1 dpf (p-value <0.0001). DrLITR 15.1 had statistically significant upregulation at 0 hpf, 4 dpf and 5 dpf (p-value <0.05). Most of the genes tested were also present in unfertilized eggs (i.e. 0 hpf), which had similar expression levels as the 1 hpf time point for each respective gene (Figures 5.1, 5.4, 5.5, 5.7, 5.8, 5.10). However, IL8, IL1β, TNFα, and DrLITR 15.1 were expressed at higher levels in unfertilized eggs than what was detected at 1 hpf (Figures 5.2, 5.3, 5.6 and 5.9).

5.2.2 Examination of DrLITR expression in adult fish using a visceral cavity-based inflammation model

Adult fish were injected with either zymosan or PBS (i.e. negative control) to examine the effect of zymosan on DrLITR expression. Fish were dissected at 3, 6, 8, 12 and 24 hours post injection and the viscera of the fish were removed. IL1β was used as the positive control. Compared to PBS, zymosan injection upregulated IL1β transcript levels significantly at 3,6, 8 and 12 hours post injection (Figure 5.11). In all cases, p-values of < 0.05 were considered significant. DrLITR 1.1 was significantly upregulated at 12 hours post zymosan exposure (Figure 5.12). DrLITR 1.2 was significantly upregulated at 3, 6 and 12 hours post zymosan injection (Figure 5.13). DrLITR 15.1 displayed no statistical significance post zymosan exposure at any time point (Figure 5.14). DrLITR 23.1 was downregulated at 24 hours post zymosan injection (Figure 5.15). A T-test was used to calculate statistical significance on log-transformed (log base 2) data of adult samples. This was done to normalize the data as it cannot be assumed that the data follows normal distribution.

5.3 DISCUSSION

In this chapter, I reported on the establishment of a qPCR-based assay that can be used to easily monitor the expression levels of DrLITRs during ontogeny and adulthood. I developed this assay mainly to study DrLITR expression profiles and used other innate immune markers as a benchmark to compare the expression of these novel receptors. I have quantified the expression of DrLITRs and demonstrated that each DrLITR has unique upregulation/downregulation patterns during embryogenesis. The same trend was also observed for adults exposed to zymosan. Interestingly, DrLITRs do not mirror the expression

patterns of the inflammatory cytokines nor do they follow similar patterns with each other. Similarly, in adults exposed to zymosan, DrLITRs do not follow the same pattern as IL1β. As a matter of fact, they do not even follow the same pattern as each other. It seems that each innate immune marker has its own molecular signature that it displays during embryogenesis and during inflammation.

I chose to replicate the results from Oyarbide et al. (2012) as they had tested four key innate immune genes (i.e. TLR22, MPO, TNFα, IL1β) throughout development. The general trends in expression that I observed correlated with the trend of the published results. For example, IL1β is upregulated before 1 dpf while TLR22, MPO, TNFα are downregulated. My results follow these trends. The actual numeric fold changes are different but that is to be expected as the embryos I used differ from the embryos that were used in the other study. The same reasoning can also be applied for the differences I observed in my three separate trials for TLR22, TNFα and DrLITR 15.1. In each trial, I used 50 embryos for each time point and therefore, the observed variability in gene expression levels may be a result of the fact that I am using different batches of embryos bred from different adults. Therefore, the variability in relative transcript abundance is expected. Nonetheless, apart from DrLITRs, I created developmental profiles for six innate immune markers. The rationale behind this was that I wanted to sample immune genes from various components of the innate immune system. I chose to pursue TLR22, MPO, TNFα and IL1β as I wanted to replicate the published results, but there were other reasons to analyze their expression. Specifically, I wanted to choose key players of the inflammatory system (i.e. TNFα, IL8 and IL1β). For example, TNFα and IL1β are key pro-inflammatory cytokines that are also involved in pathogen clearance, while IL8 is a

chemokine that activates chemotaxis and recruits immune cells to sites of infection (Grayfer and Belosevic, 2012; Havixbeck and Barreda, 2015). MPO is involved in the innate immune process of degranulation and is released by neutrophils to kill microbes (Morel,Doussiere and Vignais, 1991). TLR22 is a fish-specific innate immune receptor that has been implicated in recognition of bacterial RNA (Samanta *et al.*, 2014). C6 is a key mediator of the complement system and is part of the membrane attack complex that essentially lyses pathogens (Sarma and Ward, 2011). To summarize, I was able to replicate published results using selected innate immune markers, which validates the qPCR protocol used and, therefore, allowed me to further examine DrLITR expression patterns.

Generally, zebrafish embryos are not released into a sterile environment. On the contrary, zebrafish eggs are fertilized externally and are exposed to an environment filled with pathogens. This requires the immediate presence of innate immune markers to contribute to fish survival. According to my data, mediators of the complement system (C6), inflammatory system (IL1β, MPO, TNFα) and immune receptor families (TLR22) are present as early as 1 hpf. DrLITRs also follow this general trend. It seems these are all maternally sourced, which appears to be a common occurrence in zebrafish. For example, there is great maternal control over embryogenesis (reviewed by Lindeman and Pelegri, 2010); the cytoplasm inherited by the embryo is maternally sourced and is known as germ plasm (Wylie, 2000; Cinalli, Rangan and Lehmann, 2008). This specialized cytoplasm contains mRNA that the mother specifically passes down to its offspring for the purpose of aiding development. My data shows that key innate immune components are deliberately being passed down from mother to embryo suggesting that they may have developmental roles. It is also possible that these maternally sourced

immune genes could be used by the embryo for defense against microbes. The actual reason is unknown, but the fact that they are maternally inherited does suggest that they may have a key role during zebrafish development.

In contrast, IL8 was not detected at 1 hpf and 6 hpf but it was detected in unfertilized eggs. This suggests that the mother does pass down the mRNA encoding for IL8, however, it is not detected early in development. It is possible that this transcript is somehow degraded due to post-transcriptional modifications. For instance, microRNAs (miRNAs), more specifically, miR-430 family of miRNAs, are heavily implicated during zebrafish embryogenesis (Giraldez *et al.*, 2005). miRNAs are involved in reducing mRNA stability via deadenylation (i.e. shortening of the poly-A tail), which results in RNA degradation (Wu, Fan and Belasco, 2006). In addition, zebrafish mutants that lack mature miRNAs show morphological defects and high mortality by day 5 of embryogenesis (Giraldez *et al.*, 2005). Without miR-430 regulation of maternal RNA, it is possible that maternal mRNAs are not degraded and are translated into proteins that accumulate and cause these defects (Giraldez *et al.*, 2006). Therefore, the miRNA regulation of maternal factors is important to ensure survival of the embryo and prevent defects in morphogenesis. Interestingly, miR-430 is not maternally inherited by the embryo rather it is expressed during early embryogenesis. This suggests that the embryo is also in control of its own development as it is able to regulate mRNA transcripts without maternal interference (Giraldez *et al.*, 2005).To summarize, there are maternal mRNA transcripts that are inherited by the embryo that potentially serve developmental roles. However, the embryo can also regulate the degradation of these transcripts through post-transcriptional modifications. Altogether, it

seems that both maternal and embryonic factors exert control over and contribute to the fate of embryogenesis.

In addition to maternal inheritance in early embryogenesis, my data suggests that there are considerable fluctuations in expression before and after 1 dpf for all the genes tested. For example, C6, MPO, TLR22, IL8, TNFα and DrLITR 15.1 have low levels of expression before 1 dpf. In comparison, IL1β, DrLITR 1.1, DrLITR 1.2 and DrLITR 23.1 have high levels of expression before 1 dpf. These fluctuations can be explained by the co-regulation of mRNA transcripts via zygote initiation of transcription and the activity of miR-430. More specifically, the zygote is able to initiate transcription during the 64-cell stage (~ 2 hpf) as miR-430 is transcribed by the embryo (Kimmel *et al.*, 1995; Giraldez *et al.*, 2006). In other words, miR-430 is one of the first transcripts to be expressed by the embryo (Giraldez *et al.*, 2006; Heyn *et al.*, 2014). As a result, maternal mRNA is degraded by miR-430 while the zygote begins to initiate transcription of its own mRNA. Therefore, before 2 hpf, the embryo is mostly dependent on maternal RNA for survival. It is possible that IL1β, DrLITR 1.1, DrLITR 1.2 and DrLITR 23.1 are rapidly degraded by miR-430 and their expression begins to stabilize after the zygote takes complete control of transcription. Conversely, C6, MPO, TLR22, IL8, TNFα and DrLITR 15.1 are not passed on to embryos in high levels, therefore, the embryos begin to transcribe its own to balance the low amount of transcript passed on by the mother. My data also suggests that most of the genes tested have stabilized their expression post 1 dpf. In other words, when compared to 1 dpf, the expression levels of 2-7 dpf are no longer statistically significant. Interestingly, 1 dpf is also when macrophages are detected in the embryo and by 30 hpf, macrophages are able to protect the embryo from bacterial infections (Herbomel, Thisse and Thisse, 1999). This further supports

the importance of innate immunity as a crucial line of defense in the early stages of development. C6, MPO, IL8, TNFα, TLR22 and IL1β are involved in the innate immune system and are known to be involved in cell effector responses (Muller-Eberhard, 1986; Sunyer and Lambris, 2001; Sarma and Ward, 2011; Grayfer and Belosevic, 2012; Samanta *et al.*, 2014). LITRs, on the other hand, are found to be expressed on macrophages (Stafford *et al.*, 2006). Therefore, it is possible that once macrophages are detected, these genes might possibly take on a more defined role as cell surface-expressing receptors so their expression levels stabilize. Additionally, neutrophils are also detected after 33 hpf and perhaps this also contributes to the stabilization of these innate immune markers (Le Guyader *et al.*, 2008). At 4 and 5 dpf, DrLITR 15.1 is significantly upregulated, compared to 1 dpf. At 4 and 5 dpf, the embryo has hatched and starts to rapidly grow in length (Kimmel *et al.*, 1995). Compared to 1 dpf, the embryo is much larger, and it is possible that DrLITR 15.1 may have an additional role in this growth phase, in addition, to a possible immune role. These are all speculations as the roles of DrLITRs are not known but the main point is that each immune marker is differentially expressed with varying levels of expression throughout ontogeny.

In adults, I used a viscera-based inflammation model to measure the induction of the immune system. Specifically, zymosan, a known immunostimulant, was used to activate an inflammatory response in adult zebrafish by injecting it into their peritoneal cavity (Ruyra *et al.*, 2015). The viscera of the fish were used in order to be resourceful. For instance, to obtain enough RNA, ~10 fish would have to be sacrificed to acquire enough individual organs to perform experiments. In other words, if LITR expression was to be examined only in kidney, then multiple fish would need to be dissected to acquire multiple kidneys which equates to one

sample worth of RNA. The viscera contains four major organs therefore only one fish had to be sacrificed for one representative sample (Gonçalves *et al.*, 2017).

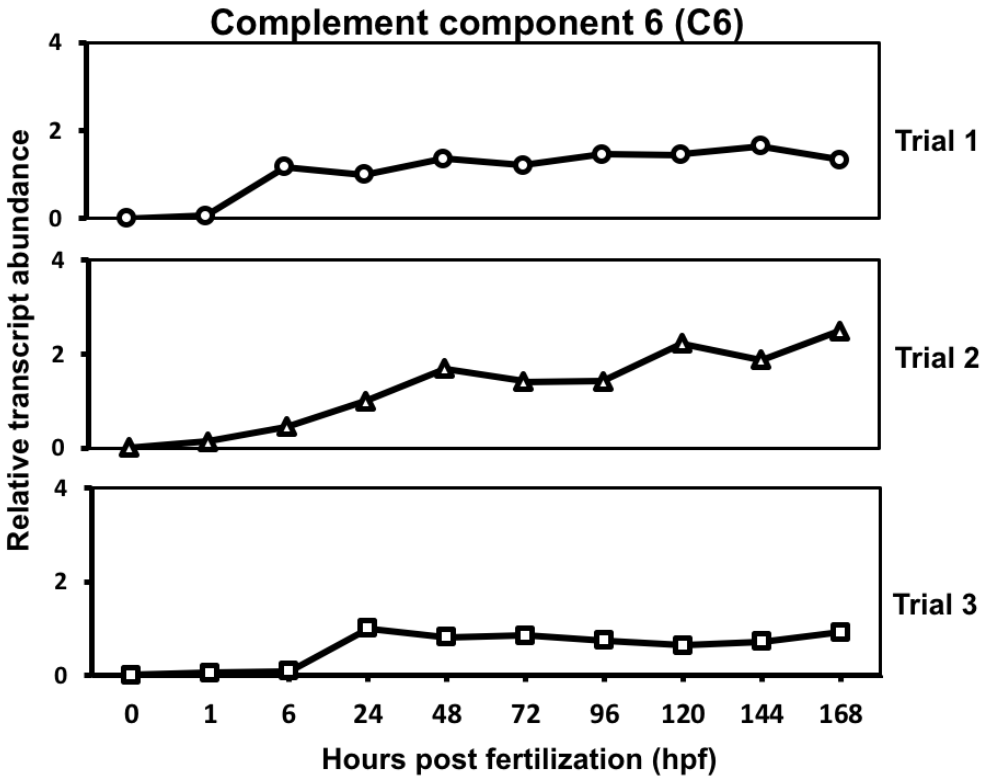
As shown in my results, IL1β is significantly upregulated at 3, 6, 8 and 12 hours post zymosan injection. This suggests that the inflammatory system has been activated and the innate immune system has been induced. Using this as a benchmark, I measured the expression levels of DrLITRs during these periods of immune system induction to observe if the expression of these receptors are affected by zymosan. My data suggests that DrLITR 1.1 was significantly upregulated at 12 hours post zymosan exposure while DrLITR 1.2 was significantly upregulated at 3, 6 and 12 hours. One reason for this upregulation could be that macrophages, expressing these receptors, are being recruited to the viscera to deal with the infection. Macrophages are known to migrate to the site of infection to perform cell effector responses, such as phagocytosis, and IpLITRs are known to be expressed on catfish macrophages (Stafford *et al.*, 2006; Duque and Descoteaux, 2014). Therefore, DrLITR 1.1 and DrLITR 1.2 may not be directly involved in this inflammatory response but rather they are being detected due to their presence on infiltrating macrophages. Additionally, DrLITRs may be involved in a process known as efferocytosis. This is the process of engulfing infected/injured host cells that are dying by apoptosis (Green, Oguin and Martinez, 2016; Elliott, Koster and Murphy, 2017). A unique aspect of efferocytosis includes the coordination of multiple receptors to engulf dying cells; this is called the “tethering and tickling” mechanism (Somersan and Bhardwaj, 2001). In general, one receptor mediates the binding of the dying cell to the surface of a phagocyte (i.e. “tethers” the cell to the phagocyte) while another receptor actually mediates the engulfment process (i.e. “tickles” the phagocyte to engulf). This mechanism can also be used to explain the expression

patterns of DrLITR 15.1 (not significantly expressed at any time point) and DrLITR 23.1 (downregulated after zymosan exposure at 24 hour time point). Perhaps, DrLITR 15.1 and DrLITR 23.1 are "tethering" receptors that are not essentially upregulated but constitutively expressed in the viscera to contribute to engulfment via communication with "tickling" receptors. Conversely, DrLITR 1.1 and DrLITR 1.2 might be "tickling" receptors that are present on phagocytes to mediate internalization and since phagocyte recruitment increases during infection, this has a direct effect on the upregulation of DrLITR 1.1 and DrLITR 1.2. To conclude, leukocyte recruitment and efferocytosis present two possible potential explanations for the expression levels of DrLITRs during zymosan induction.

When analyzing the expression of DrLITR 23.1, I observed significantly more individual variability for the PBS treatment than the zymosan treatment. For example, the expression of this receptor is higher at all time points in the PBS treatment group. One potential explanation for this could be that during immune challenge, leukocytes can alter their receptor profile. For instance, NK cells increase the expression of chemokine receptors under hypoxic conditions (Parodi *et al.*, 2018). It is possible that DrLITR 23.1 might be downregulated in leukocytes so that the leukocyte can increase its transcription for a gene/receptor that might be more useful during zymosan infection. The same explanation can also be applied to DrLITR 15.1 as it also shows downregulation at most time points. To summarize, DrLITR 1.1 and DrLITR 1.2 show high expression levels while DrLITR 15.1 and DrLITR 23.1 are relatively unaffected during zymosan-induced inflammation. The reasons for the differences in expression are currently speculative and therefore, more experiments are required to fully characterize their roles in innate immunity.

Overall, the results of this chapter demonstrate that each DrLITR shows variable expression levels throughout ontogeny and adulthood. This is the first report of expression levels of LITR-types that have been monitored during ontogeny and in the visceral cavity of adult fish after zymosan exposure.

A.


Complement component 6 (C6)
Relative transcript abundance
Trial 1
Trial 2
Trial 3
0
2
4
0 1 6 24 48 72 96 120 144 168
Hours post fertilization (hpf)

B.

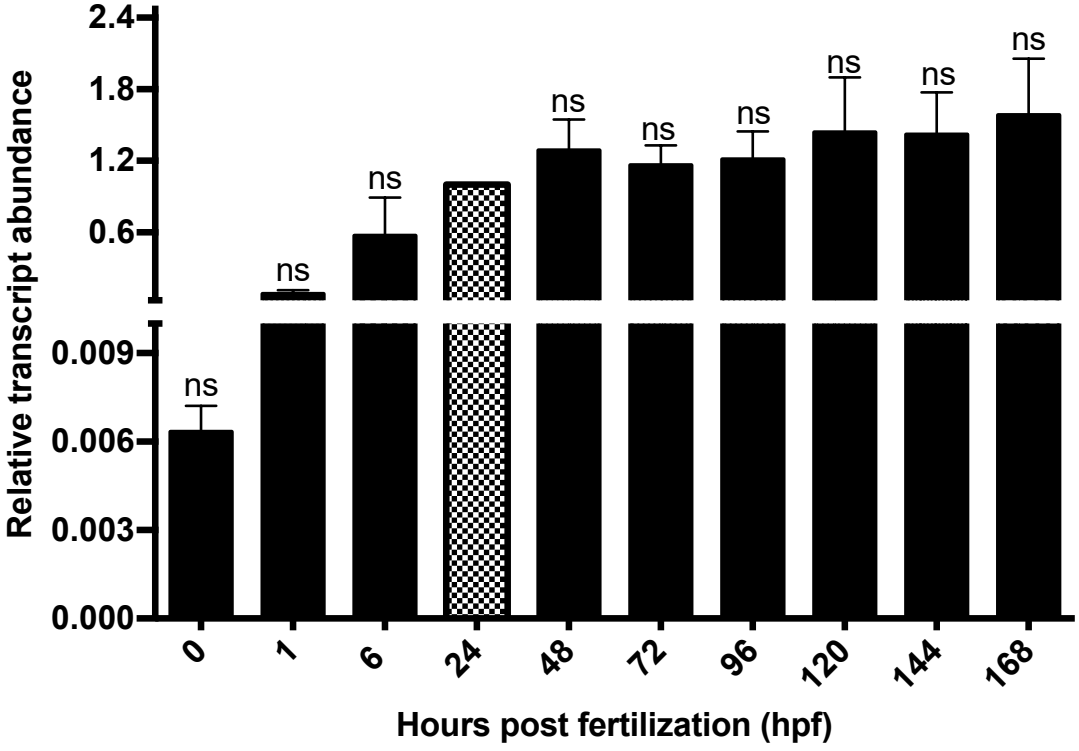
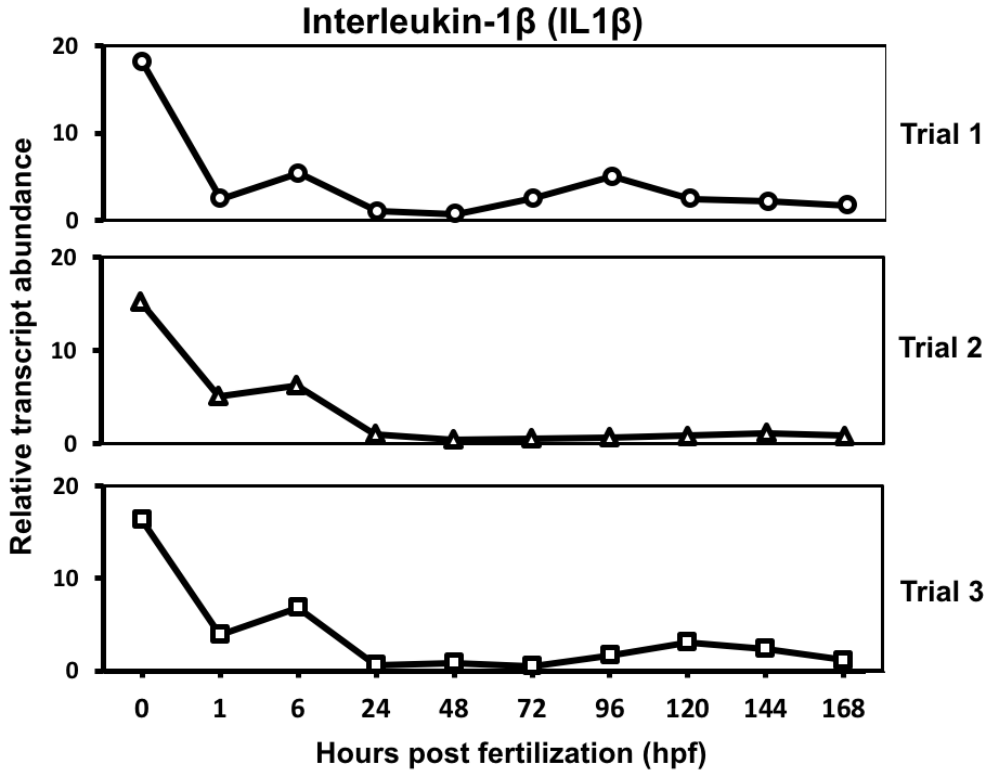

Relative transcript abundance
2.4
1.8
1.2
0.6
0.009
0.006
0.003
0.000
ns
0 1 6 24 48 72 96 120 144 168
Hours post fertilization (hpf)

Figure 5.1. Expression profile of complement component 6 (C6) during zebrafish development. Zebrafish embryos were reared in egg water (60 mg/L Instant Ocean) and were incubated at 28.5ºC. The embryos were sacrificed at specific time points by immersion in 500 µL of TriZol and cDNA was prepared. A total of 1 µg of RNA was used to synthesize cDNA in a final volume of 10 µL. The experimental time points chosen, in hours post fertilization (hpf), are: 0 (unfertilized eggs), 1, 6, 24, 48, 72, 96, 120, 144 and 168. Each experiment consisted of 50 embryos per time point. A) This experiment had a total of three replicates depicted as three trials and used to calculate B) the average expression at each respective time point. One-way ANOVA was used to test significance using 24 hpf as the reference sample. P-values of < 0.05 were considered significant. If this threshold was not met, the data was considered statistically not significant (ns). All samples are standardized to 24 hpf and β-actin.

A.


Interleukin-1β (IL1β)
Relative transcript abundance
20
10
0
Trial 1
Trial 2
Trial 3
0
1
6
24
48
72
96
120
144
168
Hours post fertilization (hpf)

B.

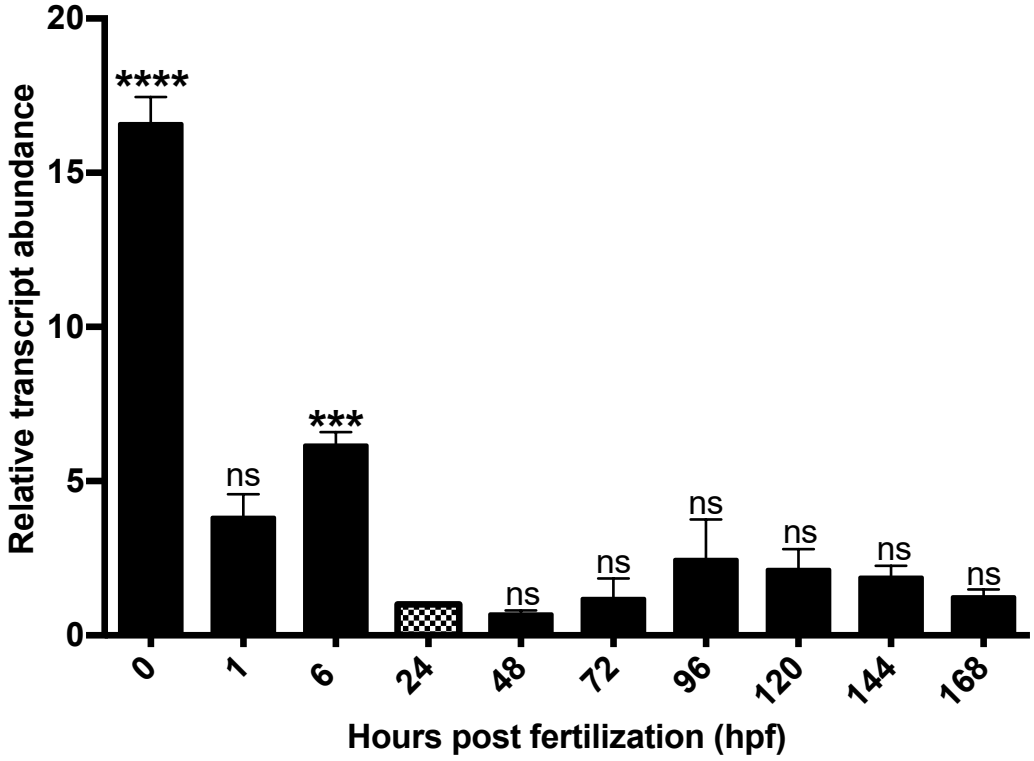
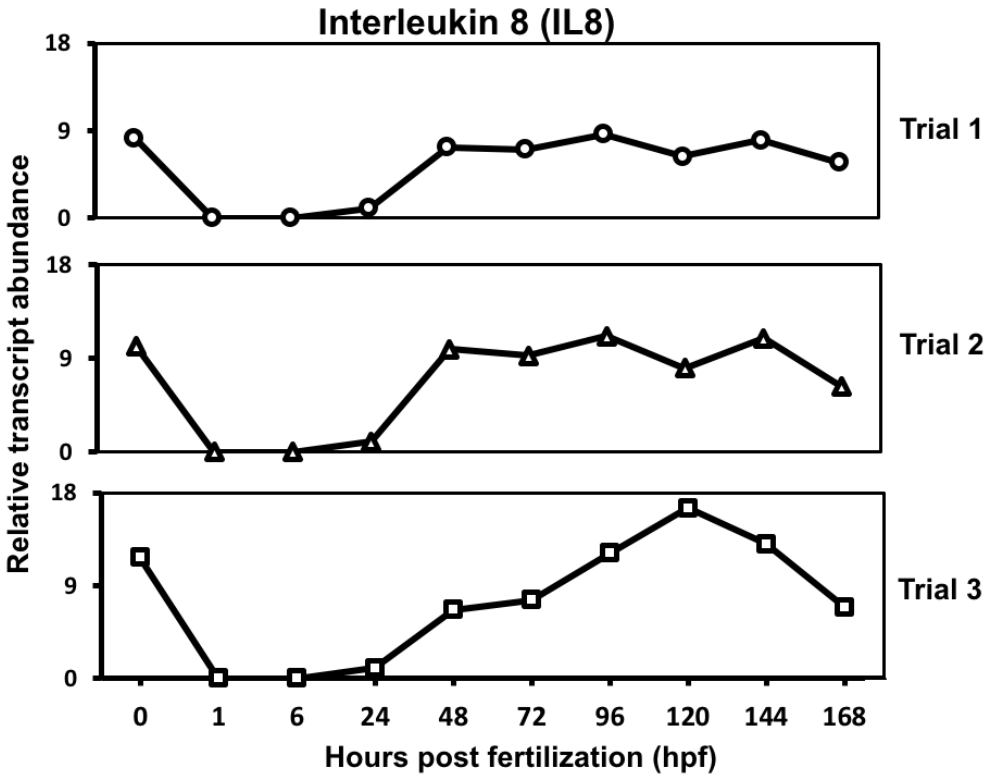

Relative transcript abundance
20
15
10
5
0
****
ns
***
ns
ns
ns
ns
ns
ns
0
1
6
24
48
72
96
120
144
168
Hours post fertilization (hpf)

Figure 5.2. Expression profile of Interleukin-1β (IL1β) during zebrafish development. Zebrafish embryos were reared in egg water (60 mg/L Instant Ocean) and were incubated at 28.5ºC. The embryos were sacrificed at specific time points by immersion in 500 µL of TriZol and cDNA was prepared. A total of 1 µg of RNA was used to synthesize cDNA in a final volume of 10 µL. The experimental time points chosen, in hours post fertilization (hpf), are: 0 (unfertilized eggs), 1, 6, 24, 48, 72, 96, 120, 144 and 168. Each experiment consisted of 50 embryos per time point. A) This experiment had a total of three replicates depicted as three trials and used to calculate B) the average expression at each respective time point. One-way ANOVA was used to test significance using 24 hpf as the reference sample. P-values of < 0.05 were considered significant and represented by asterisks. Three and four asterisks are indicative of p-values less than 0.001 and 0.0001, respectively. If the $p < 0.05$ threshold was not met, the data was considered statistically not significant (ns). All samples are standardized to 24 hpf and β-actin.

A.


Interleukin 8 (IL8)
Relative transcript abundance
18
9
0
Trial 1
Trial 2
Trial 3
0
1
6
24
48
72
96
120
144
168
Hours post fertilization (hpf)

B.

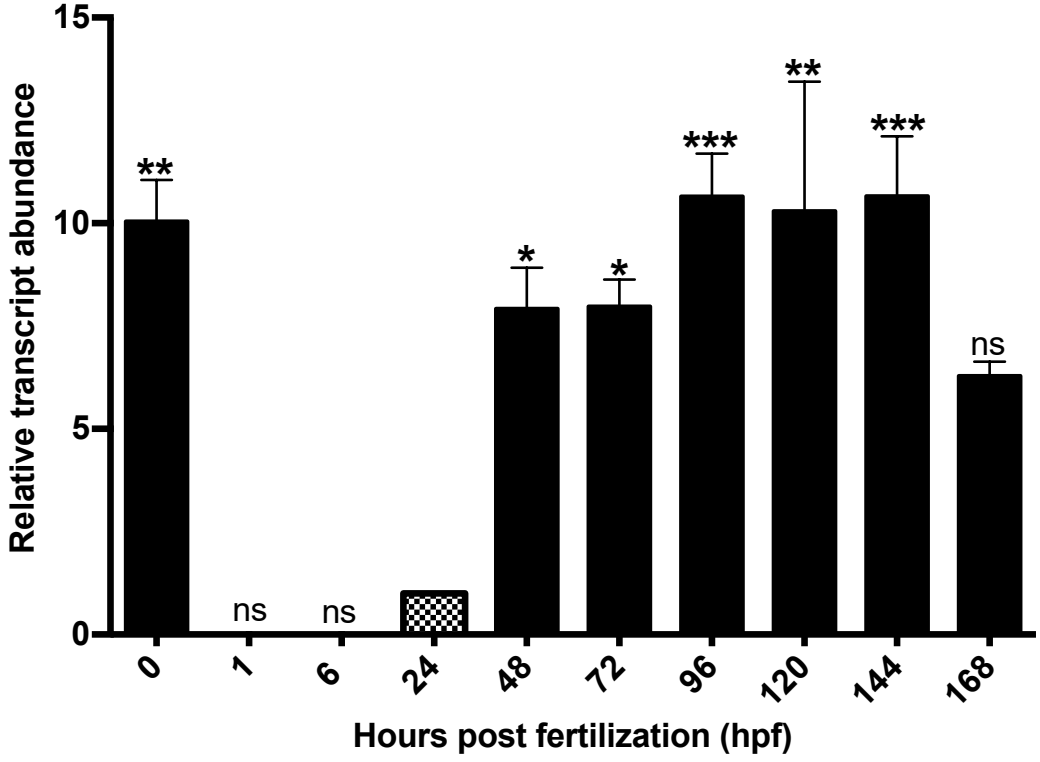
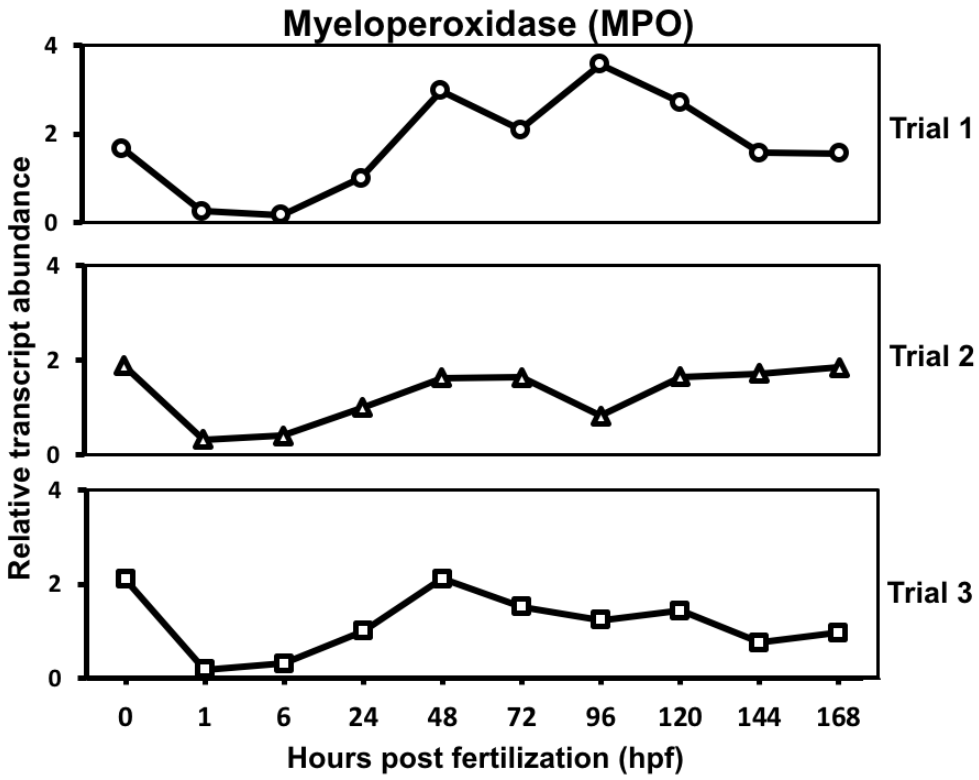

Relative transcript abundance
15
10
5
0
**
ns
ns
*
*
***
**
***
ns
0
1
6
24
48
72
96
120
144
168
Hours post fertilization (hpf)

Figure 5.3. Expression profile of Interleukin-8 (IL8) during zebrafish development. Zebrafish embryos were reared in egg water (60 mg/L Instant Ocean) and were incubated at 28.5ºC. The embryos were sacrificed at specific time points by immersion in 500 µL of TriZol and cDNA was prepared. A total of 1 µg of RNA was used to synthesize cDNA in a final volume of 10 µL. The experimental time points chosen, in hours post fertilization (hpf), are: 0 (unfertilized eggs), 1, 6, 24, 48, 72, 96, 120, 144 and 168. Each experiment consisted of 50 embryos per time point. A) This experiment had a total of three replicates depicted as three trials and used to calculate B) the average expression at each respective time point. One-way ANOVA was used to test significance using 24 hpf as the reference sample. P-values of < 0.05 were considered significant and represented by asterisks. One, two and three four asterisks are indicative of p-values of less than 0.05, 0.01 and 0.001, respectively. If the $p < 0.05$ threshold was not met, the data was considered statistically not significant (ns). All samples are standardized to 24 hpf and β-actin.

A.


Myeloperoxidase (MPO)
Relative transcript abundance
Trial 1
Trial 2
Trial 3
0
2
4
0
1
6
24
48
72
96
120
144
168
Hours post fertilization (hpf)

B.

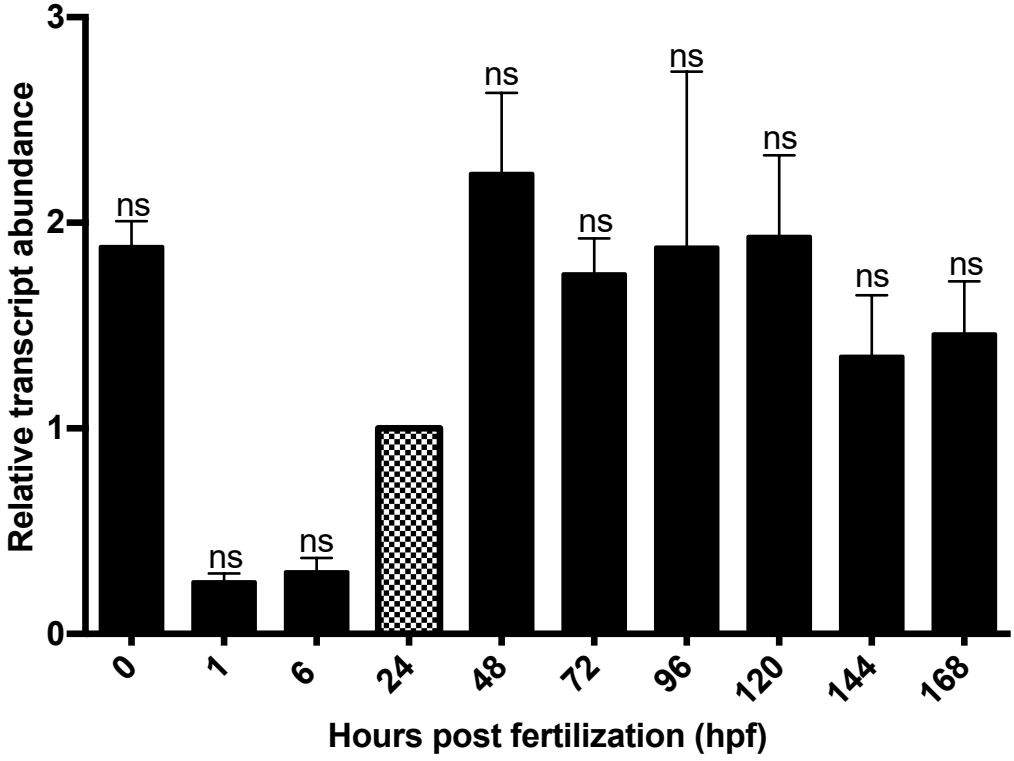
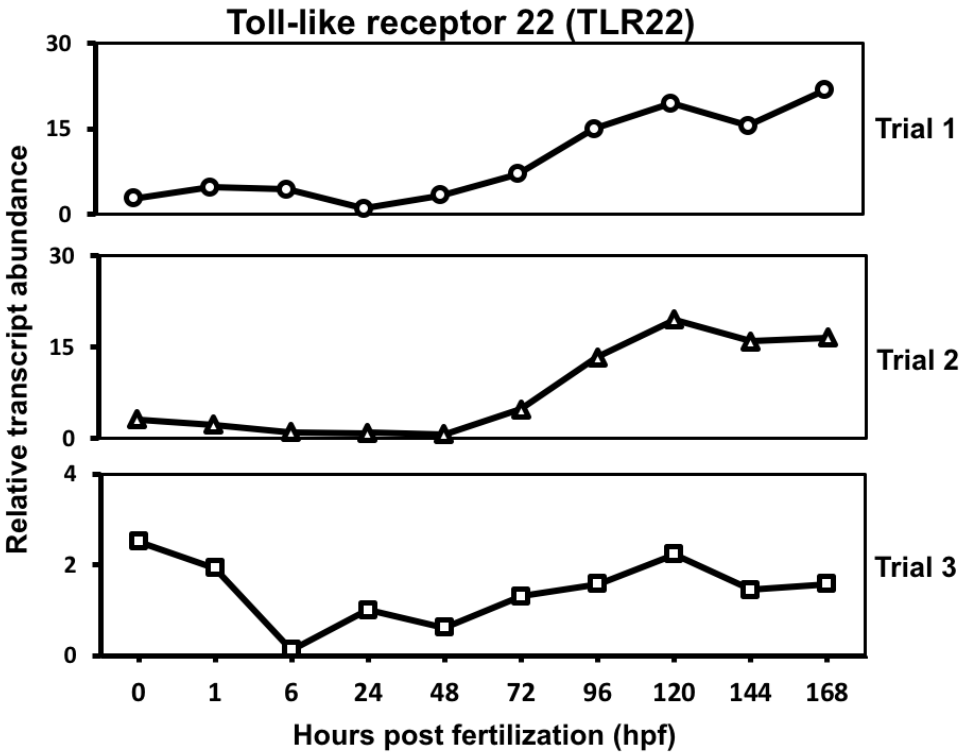

Relative transcript abundance
ns
0
1
2
3
0
1
6
24
48
72
96
120
144
168
Hours post fertilization (hpf)

Figure 5.4. Expression profile of myeloperoxidase (MPO) during zebrafish development. Zebrafish embryos were reared in egg water (60 mg/L Instant Ocean) and were incubated at 28.5ºC. The embryos were sacrificed at specific time points by immersion in 500 µL of TriZol and cDNA was prepared. A total of 1 µg of RNA was used to synthesize cDNA in a final volume of 10 µL. The experimental time points chosen, in hours post fertilization (hpf), are: 0 (unfertilized eggs), 1, 6, 24, 48, 72, 96, 120, 144 and 168. Each experiment consisted of 50 embryos per time point. A) This experiment had a total of three replicates depicted as three trials and used to calculate B) the average expression at each respective time point. One-way ANOVA was used to test significance using 24 hpf as the reference sample. P-values of < 0.05 were considered significant. If this threshold was not met, the data was considered statistically not significant (ns). All samples are standardized to 24 hpf and β-actin.

A.


Toll-like receptor 22 (TLR22)
Relative transcript abundance
30
15
0
Trial 1
30
15
0
Trial 2
4
2
0
Trial 3
0
1
6
24
48
72
96
120
144
168
Hours post fertilization (hpf)

B.

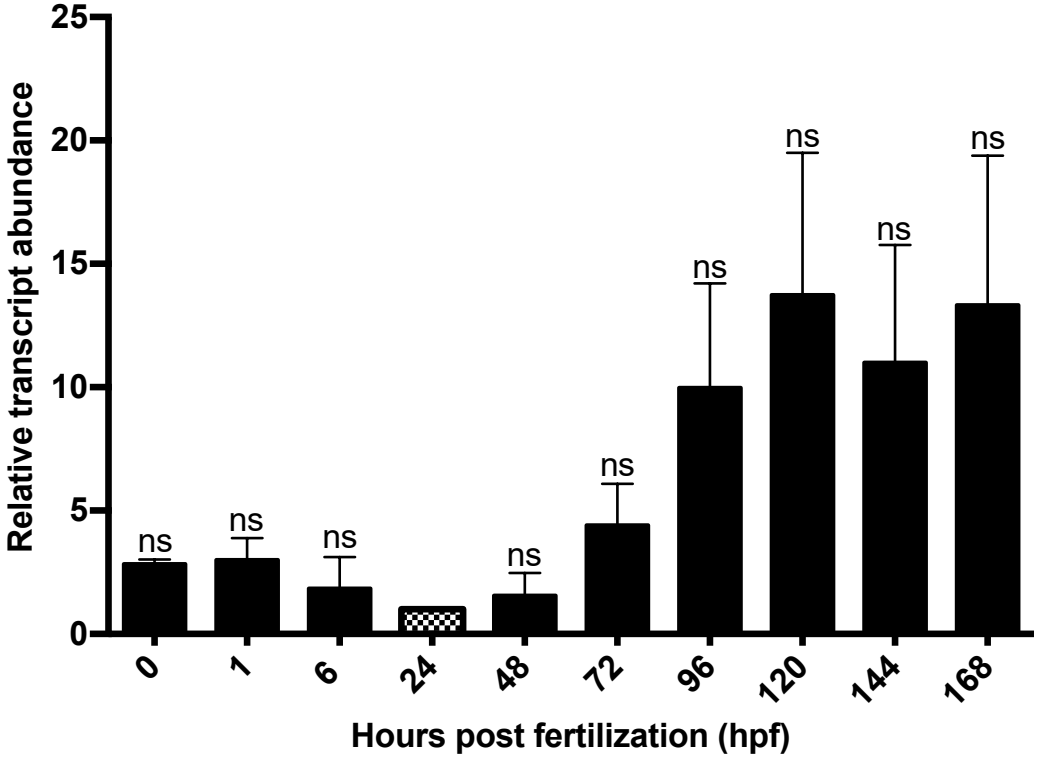
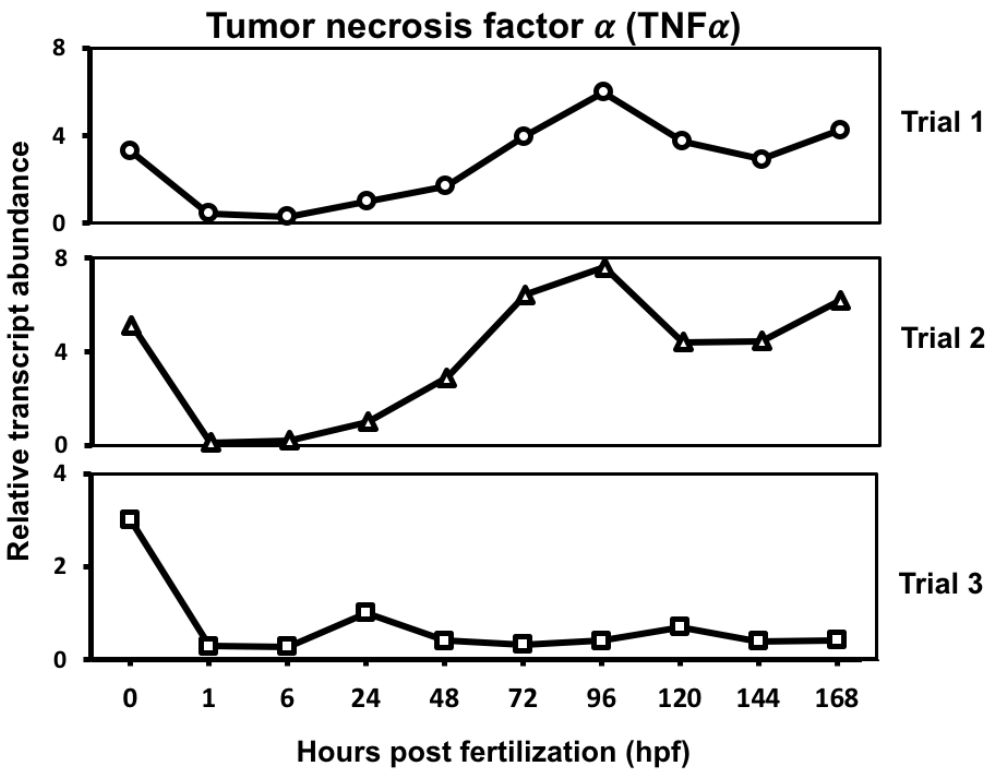

Relative transcript abundance
25
20
15
10
5
0
ns
ns
ns
ns
ns
ns
ns
ns
0
1
6
24
48
72
96
120
144
168
Hours post fertilization (hpf)

Figure 5.5. Expression profile of toll-like receptor 22 (TLR22) during zebrafish development. Zebrafish embryos were reared in egg water (60 mg/L Instant Ocean) and were incubated at 28.5ºC. The embryos were sacrificed at specific time points by immersion in 500 µL of TriZol and cDNA was prepared. A total of 1 µg of RNA was used to synthesize cDNA in a final volume of 10 µL. The experimental time points chosen, in hours post fertilization (hpf), are: 0 (unfertilized eggs), 1, 6, 24, 48, 72, 96, 120, 144 and 168. Each experiment consisted of 50 embryos per time point. A) This experiment had a total of three replicates depicted as three trials and used to calculate B) the average expression at each respective time point. One-way ANOVA was used to test significance using 24 hpf as the reference sample. P-values of < 0.05 were considered significant. If this threshold was not met, the data was considered statistically not significant (ns). All samples are standardized to 24 hpf and β-actin.

A.


Tumor necrosis factor α (TNFα)
Relative transcript abundance
Trial 1
Trial 2
Trial 3
0
4
8
2
0 1 6 24 48 72 96 120 144 168
Hours post fertilization (hpf)

B.

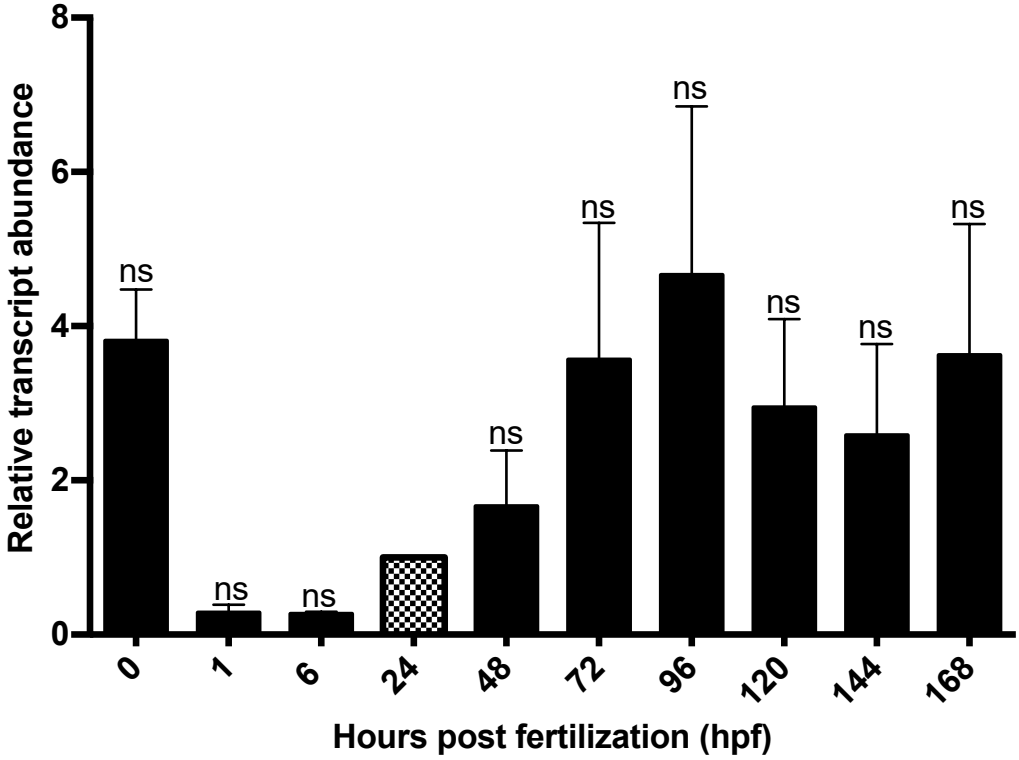
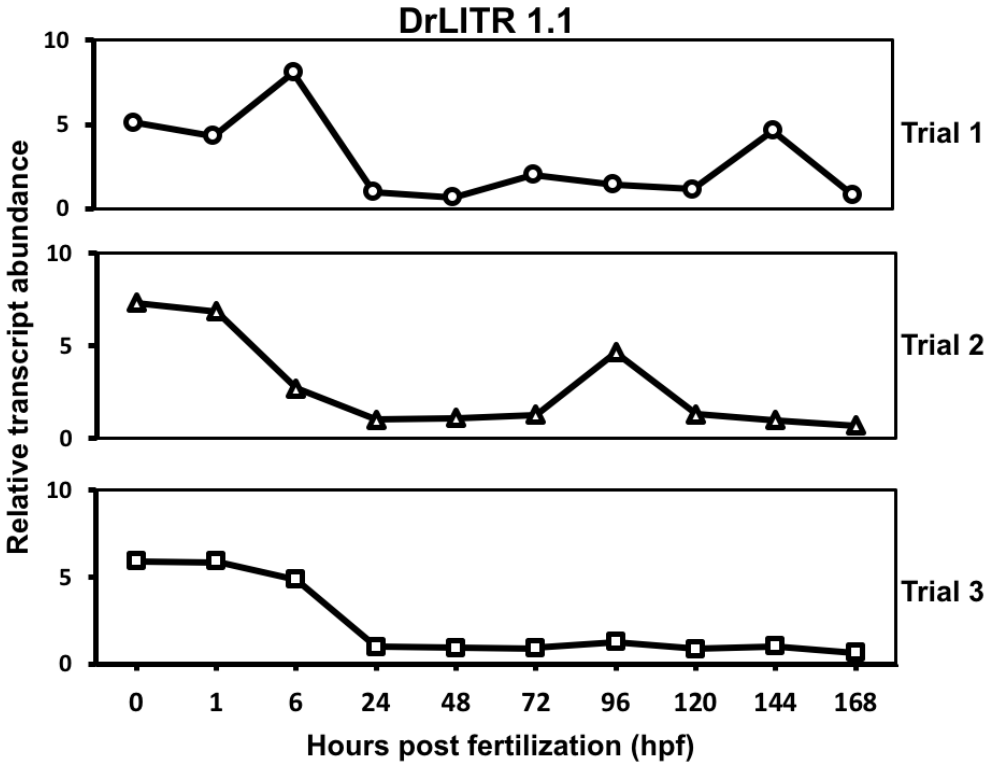

Relative transcript abundance
0
2
4
6
8
ns
0 1 6 24 48 72 96 120 144 168
Hours post fertilization (hpf)

Figure 5.6. Expression profile of tumor necrosis factor α (TNFα) during zebrafish development. Zebrafish embryos were reared in egg water (60 mg/L Instant Ocean) and were incubated at 28.5ºC. The embryos were sacrificed at specific time points by immersion in 500 µL of TriZol and cDNA was prepared. A total of 1 µg of RNA was used to synthesize cDNA in a final volume of 10 µL. The experimental time points chosen, in hours post fertilization (hpf), are: 0 (unfertilized eggs), 1, 6, 24, 48, 72, 96, 120, 144 and 168. Each experiment consisted of 50 embryos per time point. A) This experiment had a total of three replicates depicted as three trials and used to calculate B) the average expression at each respective time point. One-way ANOVA was used to test significance using 24 hpf as the reference sample. P-values of < 0.05 were considered significant. If this threshold was not met, the data was considered statistically not significant (ns). All samples are standardized to 24 hpf and β-actin.

A.


DrLITR 1.1
Relative transcript abundance
10
5
0
Trial 1
Trial 2
Trial 3
0
1
6
24
48
72
96
120
144
168
Hours post fertilization (hpf)

B.

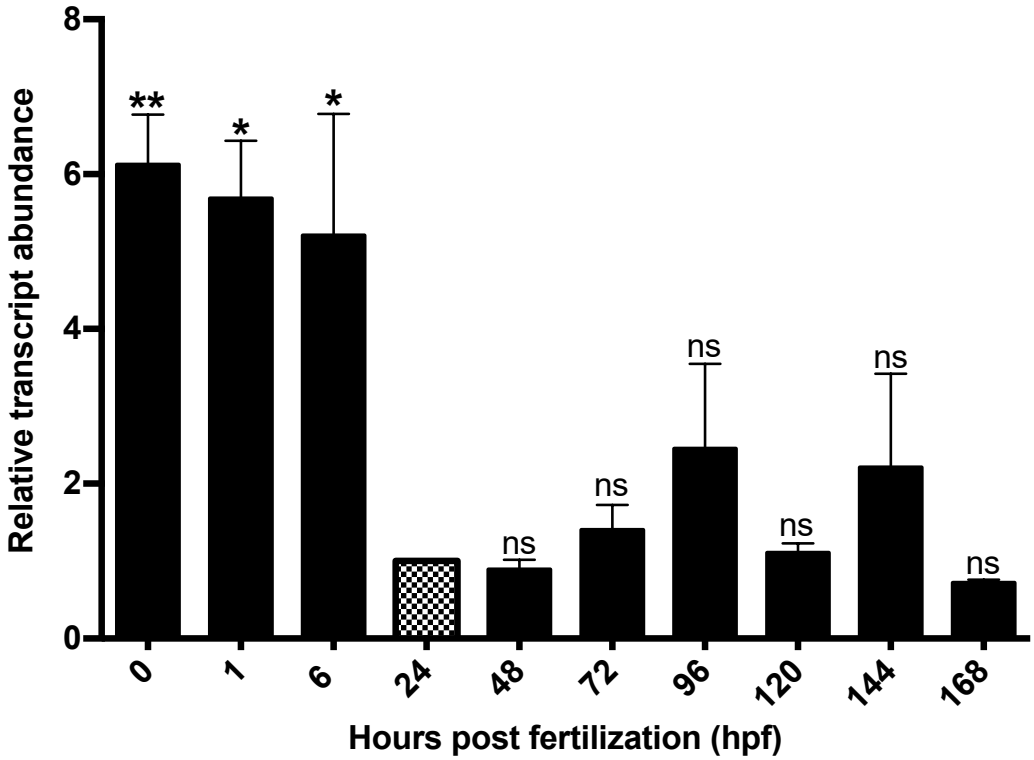
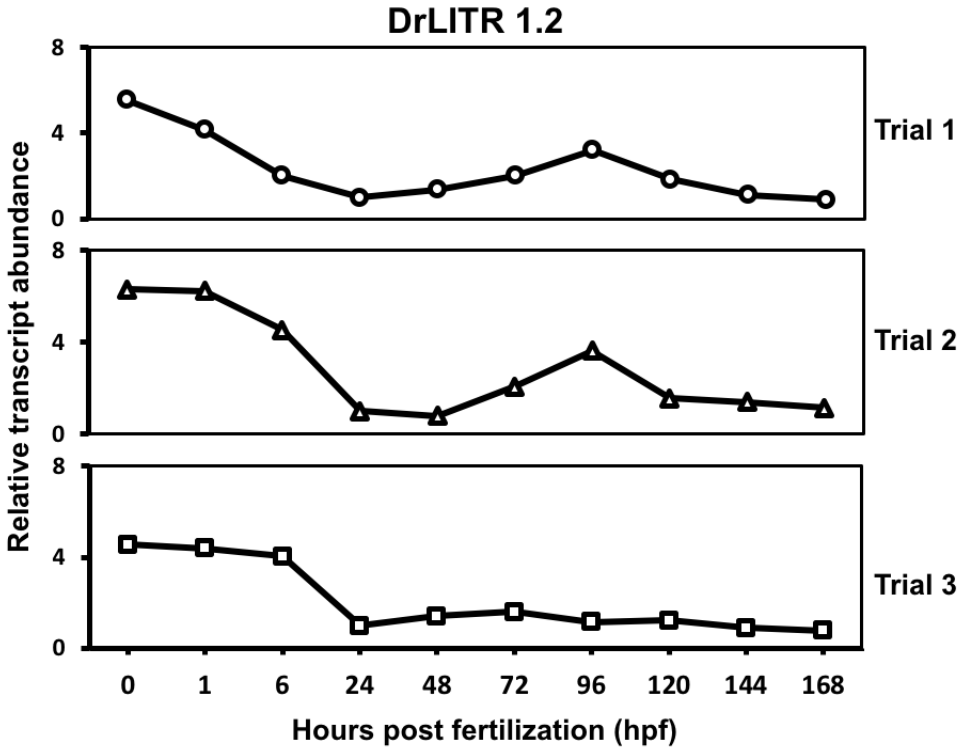

Relative transcript abundance
8
6
4
2
0
**
*
*
ns
ns
ns
ns
ns
0
1
6
24
48
72
96
120
144
168
Hours post fertilization (hpf)

Figure 5.7. Expression profile of *Danio rerio* leukocyte immune-type receptor 1.1 (DrLITR 1.1) during zebrafish development. Zebrafish embryos were reared in egg water (60 mg/L Instant Ocean) and were incubated at 28.5ºC. The embryos were sacrificed at specific time points by immersion in 500 µL of TriZol and cDNA was prepared. A total of 1 µg of RNA was used to synthesize cDNA in a final volume of 10 µL. The experimental time points chosen, in hours post fertilization (hpf), are: 0 (unfertilized eggs), 1, 6, 24, 48, 72, 96, 120, 144 and 168. Each experiment consisted of 50 embryos per time point. A) This experiment had a total of three replicates depicted as three trials and used to calculate B) the average expression at each respective time point. One-way ANOVA was used to test significance using 24 hpf as the reference sample. P-values of < 0.05 were considered significant and represented by asterisks. One and two asterisks are indicative of p-values of less than 0.05 and 0.01, respectively. If the $p < 0.05$ threshold was not met, the data was considered statistically not significant (ns). All samples are standardized to 24 hpf and β-actin.

A.


DrLITR 1.2
Relative transcript abundance
Trial 1
Trial 2
Trial 3
0
4
8
0 1 6 24 48 72 96 120 144 168
Hours post fertilization (hpf)

B.

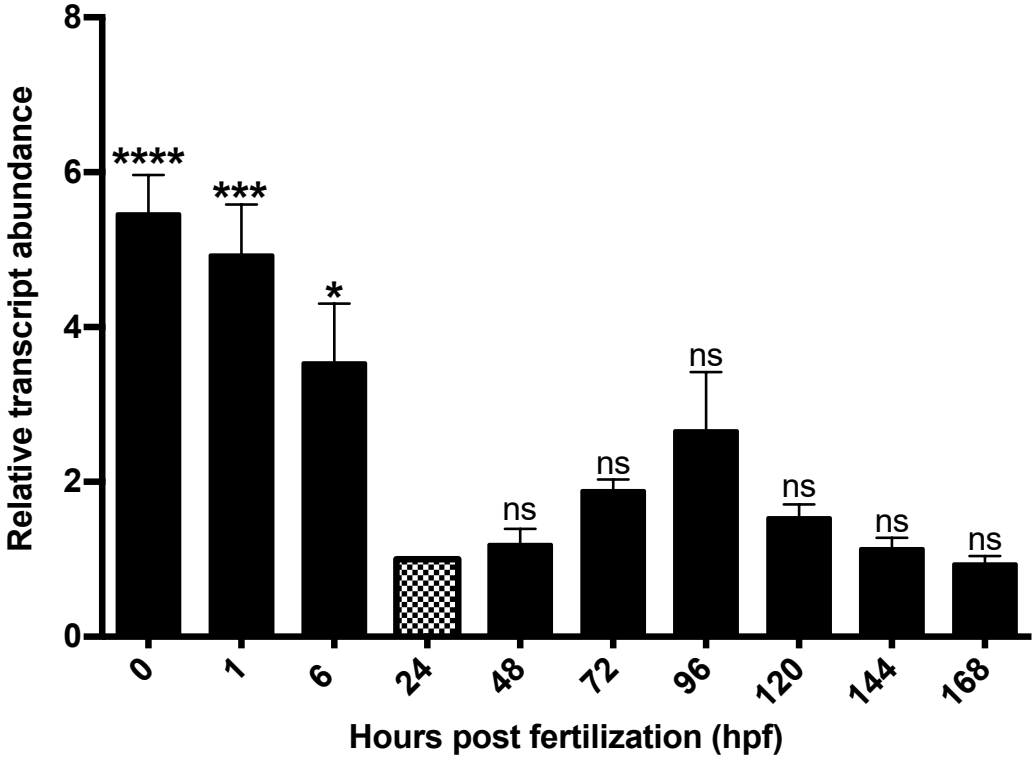
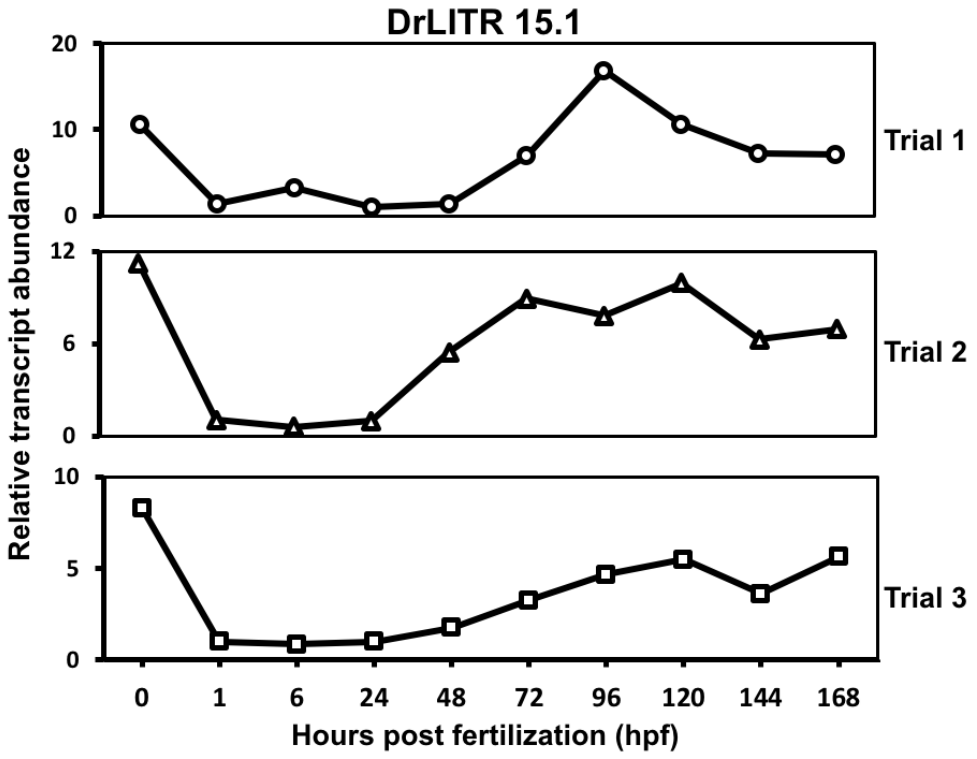

Relative transcript abundance
0
2
4
6
8
****
***
*
ns
ns
ns
ns
ns
0 1 6 24 48 72 96 120 144 168
Hours post fertilization (hpf)

Figure 5.8. Expression profile of *Danio rerio* leukocyte immune-type receptor 1.2 (DrLITR 1.2) during zebrafish development. Zebrafish embryos were reared in egg water (60 mg/L Instant Ocean) and were incubated at 28.5ºC. The embryos were sacrificed at specific time points by immersion in 500 µL of TriZol and cDNA was prepared. A total of 1 µg of RNA was used to synthesize cDNA in a final volume of 10 µL. The experimental time points chosen, in hours post fertilization (hpf), are: 0 (unfertilized eggs), 1, 6, 24, 48, 72, 96, 120, 144 and 168. Each experiment consisted of 50 embryos per time point. A) This experiment had a total of three replicates depicted as three trials and used to calculate B) the average expression at each respective time point. One-way ANOVA was used to test significance using 24 hpf as the reference sample. P-values of < 0.05 were considered significant and represented by asterisks. One and two asterisks are indicative of p-values of less than 0.05 and 0.01, respectively. If the $p < 0.05$ threshold was not met, the data was considered statistically not significant (ns). All samples are standardized to 24 hpf and β-actin.

A.


DrLITR 15.1
Relative transcript abundance
20
10
0
12
6
0
10
5
0
Trial 1
Trial 2
Trial 3
0
1
6
24
48
72
96
120
144
168
Hours post fertilization (hpf)

B.

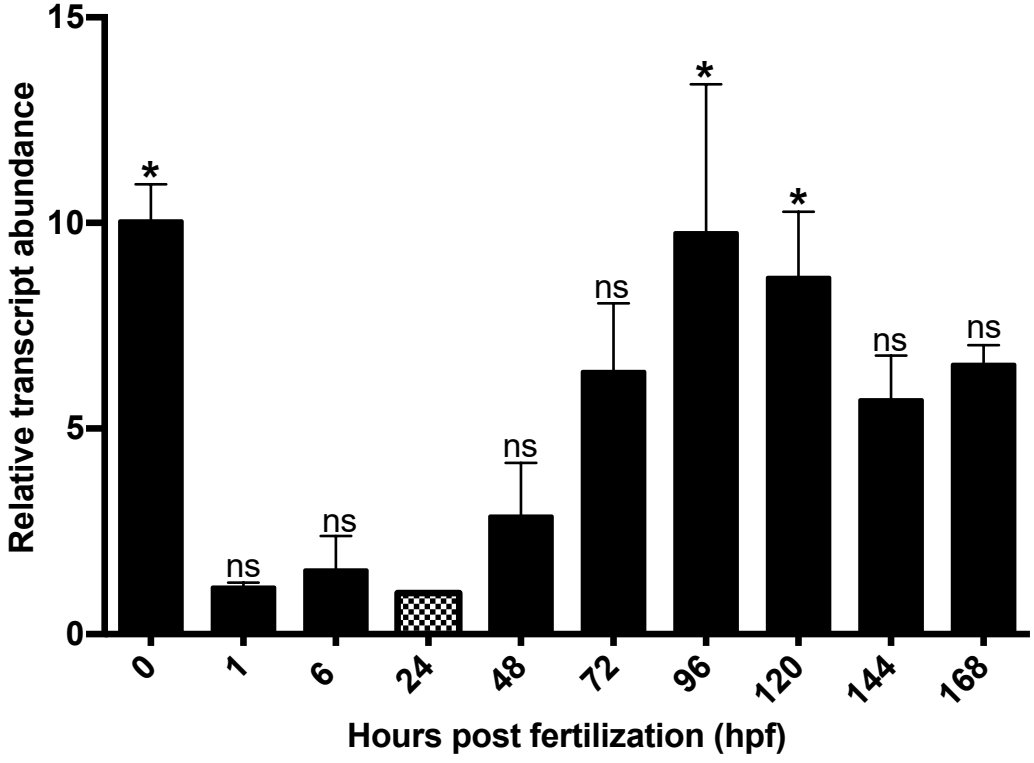
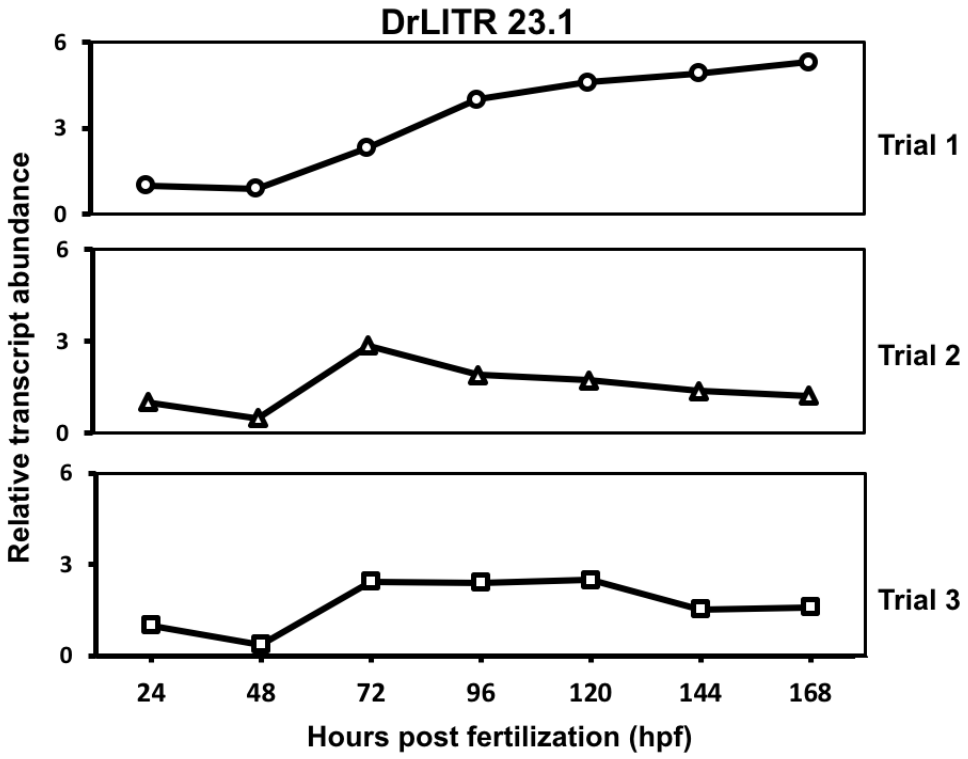

Relative transcript abundance
15
10
5
0
*
ns
ns
ns
ns
*
*
ns
ns
0
1
6
24
48
72
96
120
144
168
Hours post fertilization (hpf)

Figure 5.9. Expression profile of *Danio rerio* leukocyte immune-type receptor 15.1 (DrLITR 15.1) during zebrafish development. Zebrafish embryos were reared in egg water (60 mg/L Instant Ocean) and were incubated at 28.5ºC. The embryos were sacrificed at specific time points by immersion in 500 µL of TriZol and cDNA was prepared. A total of 1 µg of RNA was used to synthesize cDNA in a final volume of 10 µL. The experimental time points chosen, in hours post fertilization (hpf), are: 0 (unfertilized eggs), 1, 6, 24, 48, 72, 96, 120, 144 and 168. Each experiment consisted of 50 embryos per time point. A) This experiment had a total of three replicates depicted as three trials and used to calculate B) the average expression at each respective time point. One-way ANOVA was used to test significance using 24 hpf as the reference sample. P-values of < 0.05 were considered significant and represented by one asterisk. If the $p < 0.05$ threshold was not met, the data was considered statistically not significant (ns). All samples are standardized to 24 hpf and β-actin.

A.


DrLITR 23.1
Relative transcript abundance
6
3
0
Trial 1
Trial 2
Trial 3
24
48
72
96
120
144
168
Hours post fertilization (hpf)

B.

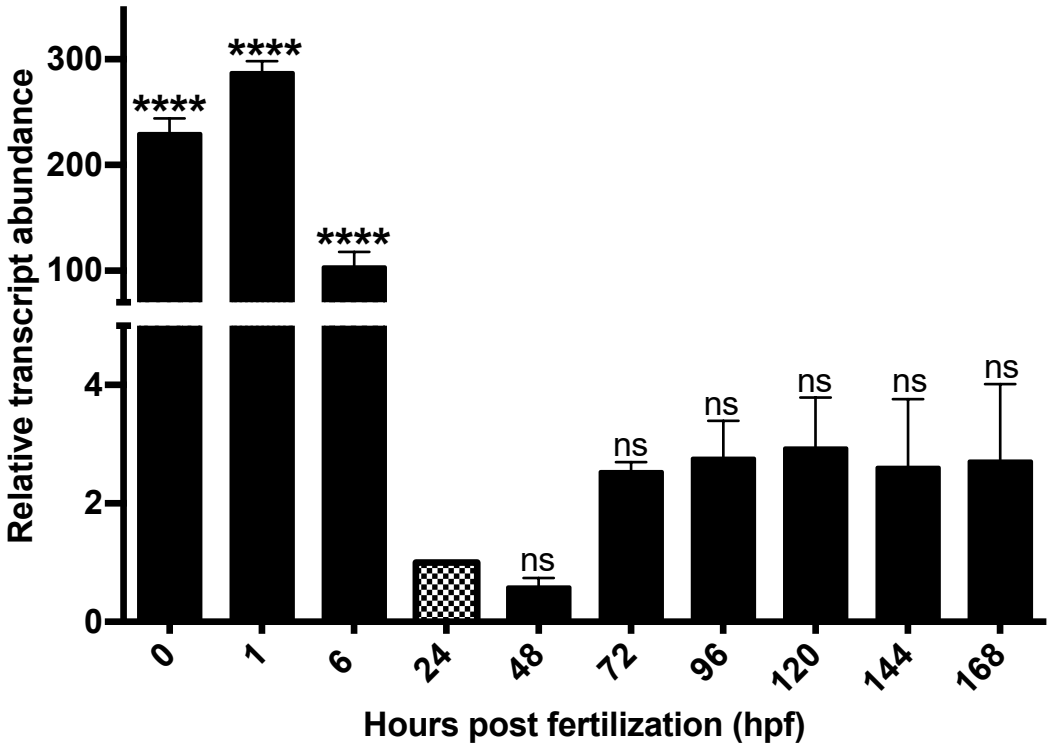

Relative transcript abundance
300
200
100
4
2
0
****
****
****
ns
ns
ns
ns
ns
0
1
6
24
48
72
96
120
144
168
Hours post fertilization (hpf)

Figure 5.10. Expression profile of *Danio rerio* leukocyte immune-type receptor 23.1 (DrLITR 23.1) during zebrafish development. Zebrafish embryos were reared in egg water (60 mg/L Instant Ocean) and were incubated at 28.5ºC. The embryos were sacrificed at specific time points by immersion in 500 µL of TriZol and cDNA was prepared. A total of 1 µg of RNA was used to synthesize cDNA in a final volume of 10 µL. The experimental time points chosen, in hours post fertilization (hpf), are: 0 (unfertilized eggs), 1, 6, 24, 48, 72, 96, 120, 144 and 168. Each experiment consisted of 50 embryos per time point. A) This experiment had a total of three replicates depicted as three trials and used to calculate B) the average expression at each respective time point. One-way ANOVA was used to test significance using 24 hpf as the reference sample. P-values of < 0.05 were considered significant and represented by asterisks. Four asterisks are indicative of p-value < 0.0001. If the $p < 0.05$ threshold was not met, the data was considered statistically not significant (ns). All samples are standardized to 24 hpf and β-actin. The 0 hpf, 1 hpf and 6 hpf have not been included in (A) as the data was on a different scale but the average is depicted in (B).

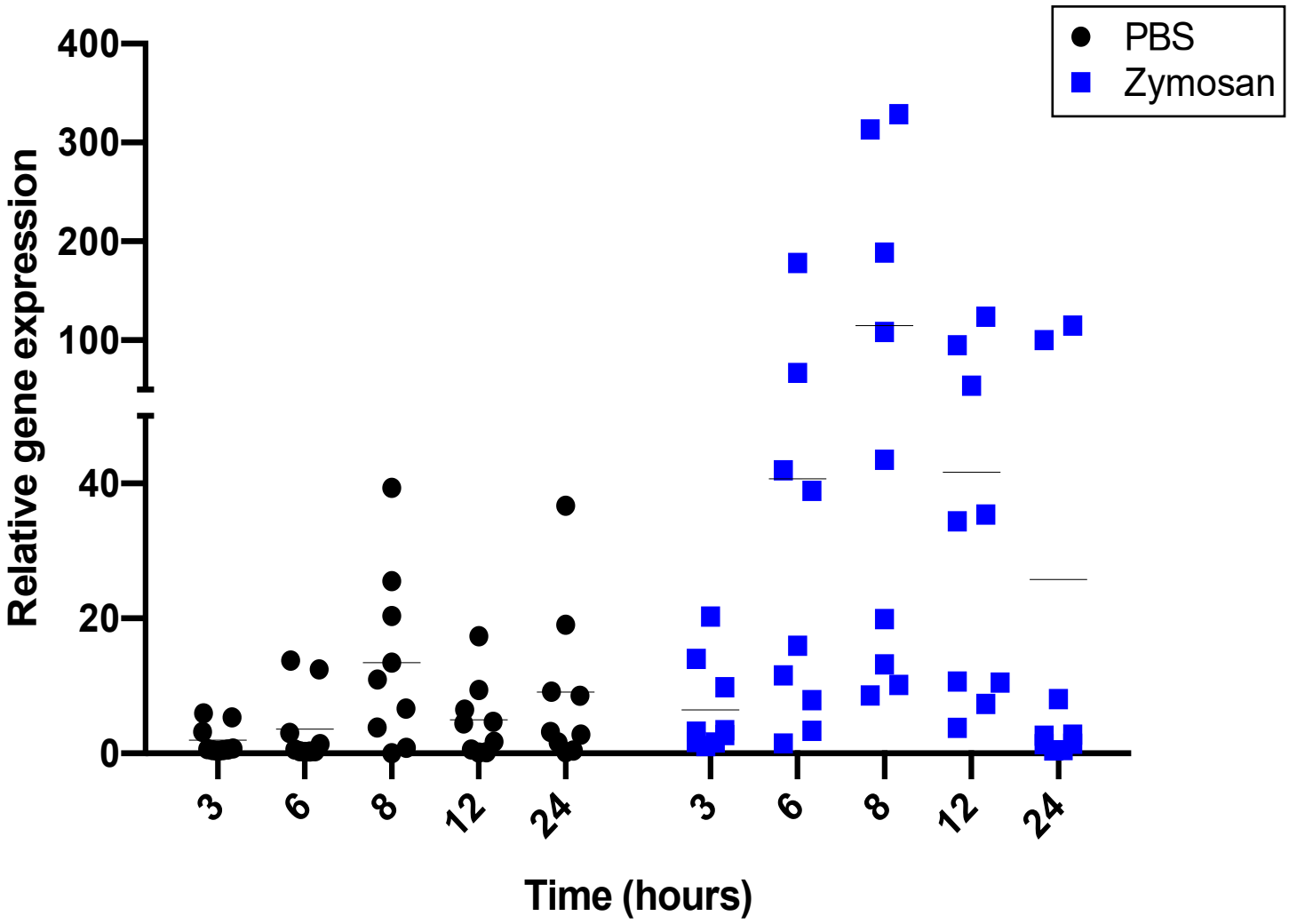

PBS
Zymosan
Relative gene expression
400
300
200
100
40
20
0
3
6
8
12
24
3
6
8
12
24
Time (hours)

Figure 5.11. Expression profile of Interleukin-1β (IL1β) in adults after stimulation with zymosan. Adult zebrafish were intraperitoneally injected with either 20 µL of 1X phosphate buffered saline (PBS) or 20 µL of zymosan A (1 mg/mL). The fish were then returned to their tanks and dissected at specific time intervals. The experimental time points chosen, in hours, were: 3, 6, 8, 12 and 24. Fish were immersed and euthanized in 200 mg/L of tricaine followed by decapitation. The viscera of the fish were obtained which contains the liver, intestines, pancreas and spleen of the fish. Each dot/square represents one viscera from one fish. Three additional fish were injected with 1X PBS and sacrificed immediately to serve as the reference sample. All samples are standardized to the reference sample and β-actin. A T-test was used to calculate statistical significance on log-transformed (log base 2) data of all samples. P-values were < 0.05 for the zymosan treatments at the 3 hour and 8 hour time points, when compared to PBS. P-values were < 0.01 for the zymosan treatments at the 6 hour and 12 hour time points, when compared to PBS. The 24 hour time point was considered statistically insignificant as the $p < 0.05$ threshold was not met.

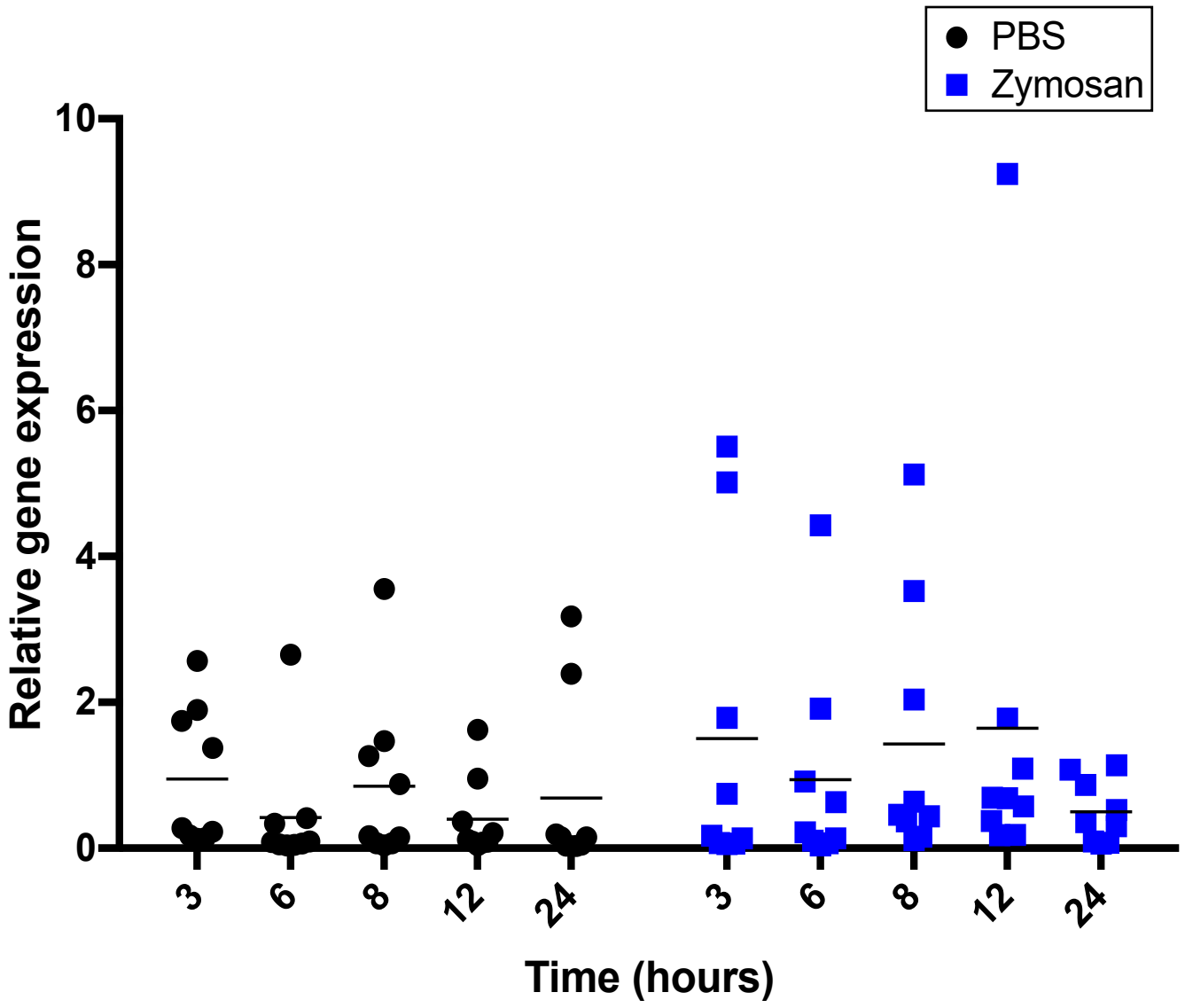

PBS
Zymosan
Relative gene expression
10
8
6
4
2
0
3
6
8
12
24
3
6
8
12
24
Time (hours)

Figure 5.12. Expression profile of *Danio rerio* leukocyte immune-type receptor 1.1 (DrLITR 1.1) in adults after stimulation with zymosan. Adult zebrafish were intraperitoneally injected with either 20 µL of 1X phosphate buffered saline (PBS) or 20 µL of zymosan A (1 mg/mL). The fish were then returned to their tanks and dissected at specific time intervals. The experimental time points chosen, in hours, were: 3, 6, 8, 12 and 24. Fish were immersed and euthanized in 200 mg/L of tricaine followed by decapitation. The viscera of the fish were obtained which contains the liver, intestines, pancreas and spleen of the fish. Each dot/square represents one viscera from one fish. Three additional fish were injected with 1X PBS and sacrificed immediately to serve as the reference sample. All samples are standardized to the reference sample and β-actin. A T-test was used to calculate statistical significance on log-transformed (log base 2) data of all samples. P-values were < 0.05 for the zymosan treatments at the 12 hour time point, when compared to PBS. The 3, 6, 8 and 24 hour time point were considered statistically insignificant as the $p < 0.05$ threshold was not met.

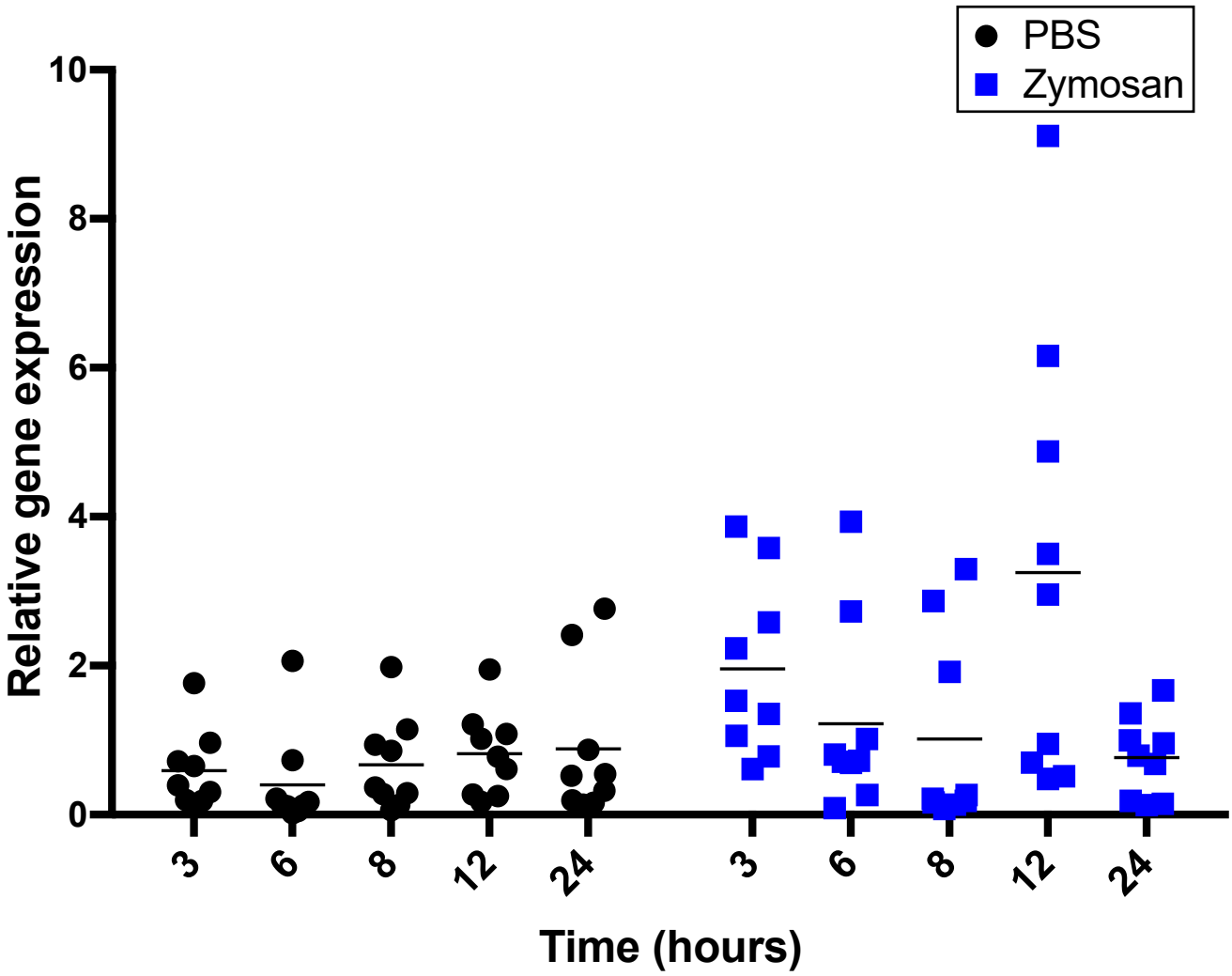

PBS
Zymosan
Relative gene expression
10
8
6
4
2
0
3
6
8
12
24
3
6
8
12
24
Time (hours)

Figure 5.13. Expression profile of *Danio rerio* leukocyte immune-type receptor 1.2 (DrLITR 1.2) in adults after stimulation with zymosan. Adult zebrafish were intraperitoneally injected with either 20 µL of 1X phosphate buffered saline (PBS) or 20 µL of zymosan A (1 mg/mL). The fish were then returned to their tanks and dissected at specific time intervals. The experimental time points chosen, in hours, were: 3, 6, 8, 12 and 24. Fish were immersed and euthanized in 200 mg/L of tricaine followed by decapitation. The viscera of the fish were obtained which contains the liver, intestines, pancreas and spleen of the fish. Each dot/square represents one viscera from one fish. Three additional fish were injected with 1X PBS and sacrificed immediately to serve as the reference sample. All samples are standardized to the reference sample and β-actin. A T-test was used to calculate statistical significance on log-transformed (log base 2) data of all samples. P-values were < 0.05 for the zymosan treatments at the 6 hour and 12 hour time points, when compared to PBS. P-values were < 0.01 for the zymosan treatments at the 3 hour time point, when compared to PBS. The 8 and 24 hour time points were considered statistically insignificant as the $p < 0.05$ threshold was not met.

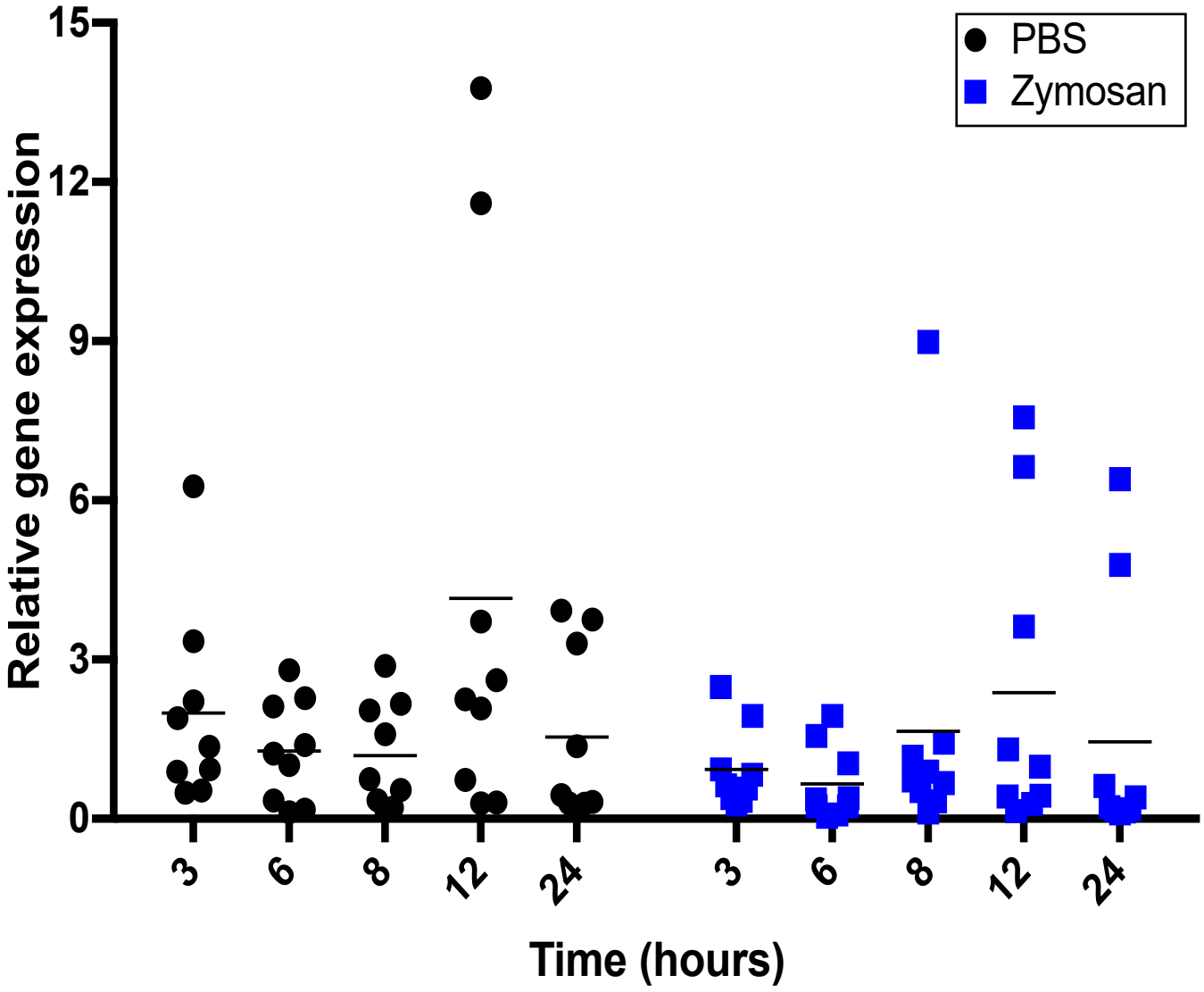

PBS
Zymosan
Relative gene expression
15
12
9
6
3
0
3
6
8
12
24
3
6
8
12
24
Time (hours)

Figure 5.14. Expression profile of *Danio rerio* leukocyte immune-type receptor 15.1 (DrLITR 15.1) in adults after stimulation with zymosan. Adult zebrafish were intraperitoneally injected with either 20 µL of 1X phosphate buffered saline (PBS) or 20 µL of zymosan A (1 mg/mL). The fish were then returned to their tanks and dissected at specific time intervals. The experimental time points chosen, in hours, were: 3, 6, 8, 12 and 24. Fish were immersed and euthanized in 200 mg/L of tricaine followed by decapitation. The viscera of the fish were obtained which contains the liver, intestines, pancreas and spleen of the fish. Each dot/square represents one viscera from one fish. Three additional fish were injected with 1X PBS and sacrificed immediately to serve as the reference sample. All samples are standardized to the reference sample and β-actin. A T-test was used to calculate statistical significance on log-transformed (log base 2) data of all samples. There was no statistical significance observed between the PBS and zymosan treatments for all time points. If the $p < 0.05$ threshold was not met, the data was considered statistically not significant (ns).

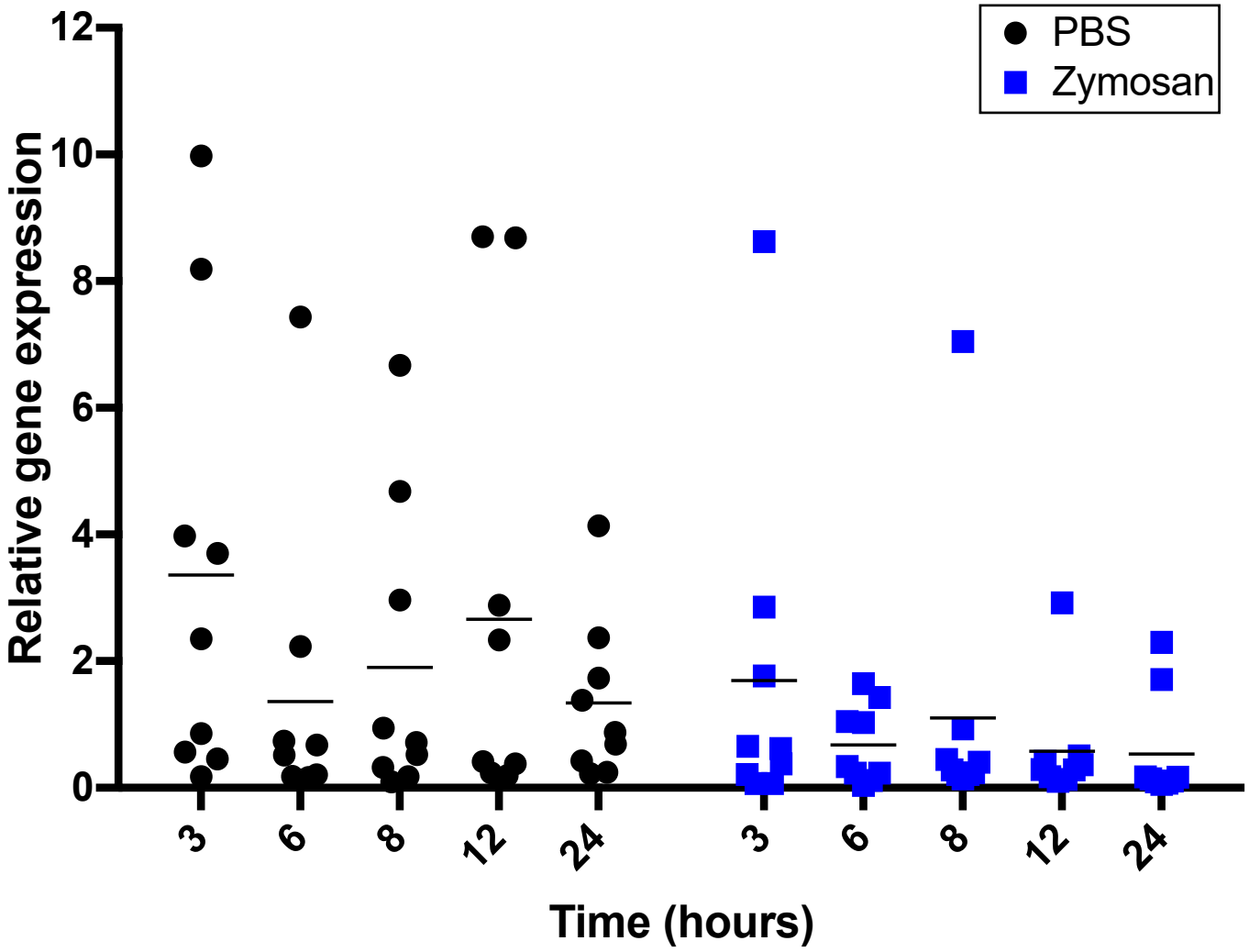

PBS
Zymosan
Relative gene expression
0
2
4
6
8
10
12
3
6
8
12
24
3
6
8
12
24
Time (hours)

Figure 5.15. Expression profile of *Danio rerio* leukocyte immune-type receptor 23.1 (DrLITR 23.1) in adults after stimulation with zymosan. Adult zebrafish were intraperitoneally injected with either 20 µL of 1X phosphate buffered saline (PBS) or 20 µL of zymosan A (1 mg/mL). The fish were then returned to their tanks and dissected at specific time intervals. The experimental time points chosen, in hours, were: 3, 6, 8, 12 and 24. Fish were immersed and euthanized in 200 mg/L of tricaine followed by decapitation. The viscera of the fish were obtained which contains the liver, intestines, pancreas and spleen of the fish. Each dot/square represents one viscera from one fish. Three additional fish were injected with 1X PBS and sacrificed immediately to serve as the reference sample. All samples are standardized to the reference sample and β-actin. A T-test was used to calculate statistical significance on log-transformed (log base 2) data of all samples. P-values were < 0.05 for the zymosan treatments at the 24 hour time point, when compared to PBS. The 3, 6, 8 and 12 hour time points were considered statistically insignificant as the $p < 0.05$ threshold was not met.

CHAPTER VI

GENERAL DISCUSSION AND FUTURE DIRECTIONS

6.1 SUMMARY OF THESIS FINDINGS

LITRs are one of the many immunoregulatory receptor families that have been discovered in teleost species, however, their expression patterns and functions *in vivo* are largely unknown. My thesis findings have set the stage for understanding the roles of LITRs during ontogeny and also during an inflammatory response in adult fish. In general, the presence of an immunoregulatory receptor-type during specific stages of an organism's lifecycle suggests the potential involvement of the receptor for a specific function. For example, CEACAMs are expressed during development and have been implicated to be involved in cell migration to integrate cells into functional organs (Kuespert, Pils and Hauck, 2006). My thesis focused on establishing zebrafish as a model for further examining DrLITR expression during development and inflammation.

LITRs were originally discovered in channel catfish over a decade ago (Stafford *et al.*, 2006). They were shown to be structurally and phylogenetically related to mammalian receptors, such as FcRLs and LRC-encoded molecules. In addition, they are mainly expressed on the surfaces of immune cells. Previous studies have also shown that certain IpLITR-types can trigger ITAM-mediated cell effector responses (Lillico *et al.*, 2015). This suggests the evolutionarily conserved nature of the regulation of immune responses via immunoregulatory receptors present in various organisms. Conversely, IpLITRs also mediate novel signalling mechanisms through which cell effector responses are activated. In addition, an inhibitory receptor is capable of activating immune responses through the differential and independent

regulation of its two CYT sections (Montgomery *et al.*, 2012). This dual behaviour (conserved and novel mechanisms) of LITRs highlight their need to be studied as these signalling pathways are either present or have the potential to be present in mammals. These novel receptors need to be characterized further to expand our knowledge about the regulation of innate immune responses.

The focus of my thesis was to establish an *in vivo* model in which expression patterns of teleost LITR-types can be further studied. Zebrafish is an established immune model as well as a developmental model (Yoder *et al.*, 2002; Traver *et al.*, 2003). A useful advantage to zebrafish is the fact that the genome is published. My project was dependent on finding potential LITR-types in a new species, so the availability of the genome was key to finding candidate LITR genes. In addition, there is no data available on the expression of LITRs during ontogeny. Zebrafish is one of the few teleost species with a relatively rapid development period (i.e.~3 months to adulthood). Additionally, zebrafish have all the major immune cell lineages as mammals, including but not limited to, macrophages, T cells, B cells and neutrophils (Yoder *et al.*, 2002; Traver *et al.*, 2003). Therefore, this teleost species can be used to study the conserved aspects of the vertebrate immune system. The relative ease of breeding and the opportunity to study gene expression during ontogeny also make zebrafish an ideal model organism to study immune system development. Zebrafish are fertilized externally so the development of the immune system can be studied immediately following fertilization. Mammals take a considerably longer time to develop and therefore, using zebrafish is a more efficient way to study the highly conserved innate immune system. There is already data available indicating when certain immune genes are expressed during development (Lam *et al.*, 2004). For instance,

IL1β is detected as early as 0.2 hpf while another major pro-inflammatory cytokine, TNFβ, is detected after 4.3 hpf (Ito *et al.*, 2008). I used these immune genes to act as positive controls in my experiments and used them as a benchmark to compare the expression levels of the newly identified DrLITRs. These were the main reasons why zebrafish was pursued for LITR research rather than other teleost species. As a result of these advantages, I was able to characterize four LITR-types during ontogeny and adulthood in zebrafish.

To summarize, I established zebrafish as a model organism in which LITRs have been identified and their expression levels quantified. More specifically, I developed a qPCR assay that can be used to study the expression of DrLITRs during any developmental stage and/or during an inflammatory response. Using this assay, I have shown that DrLITRs are expressed throughout ontogeny and adulthood as well as during zymosan exposure. I have set the stage of advancing LITR research by identifying and characterizing LITRs in zebrafish. This model organism can now be used as a tool to further elucidate the functional roles of LITRs *in vivo*.

6.1.1 Identification and molecular characterization of select DrLITRs

Based on the sequences of IpLITRs, I used BLAST and EST databases to identify some putative LITR-type transcripts in zebrafish on the Ensembl database. DrLITR 1.1 and DrLITR 1.2 have one ITAM and one ITIM in their CYT region. In the literature, there is no receptor that has been documented with the presence of both ITAM and ITIM motifs in the CYT region. This further highlights the unique structure of DrLITR 1.1 and DrLITR 1.2 and the need to functionally characterize these receptors. The question remains to be answered if these receptors act as stimulatory receptors or inhibitory ones. Additionally, these receptors may switch between activation and inhibition depending on the context of the immune stimulus. There are

immunoregulatory receptor-types that contain ITIM-like or ITAM-like motifs. For instance, FcRL5 has an ITAM-like motif and an ITIM motif and was shown to both stimulate and inhibit B-cell mediated functions in response to the availability of low and high SHP-1 levels, respectively (Zhu *et al.*, 2013). DrLITR 15.1 contains two ITIMs and one ITSM. ITIMs can stimulate and inhibit responses, as seen in IpLITR 1.1b. ITIMs and ITSMs can also show functional versatility, as seen in IpLITR 1.1b and mice LMIR3 (Izawa *et al.*, 2012). In mice, LMIR3 binds ceramide and inhibits mast cell activation via ITIM- and ITSM-dependent pathways. IpLITR 1.1b is an ITSM and ITIM containing receptor that can stimulate phagocytosis in RBL-2H3 cells (Cortes *et al.*, 2014; Lillico *et al.*, 2015). DrLITR 23.1 has no tyrosine-based motifs in its CYT region but it can associate with adaptor proteins, such as FcRγL, as seen in IpLITR 2.6b. It has been shown that this receptor can bind FcRγ-L and activate degranulation, cytokine secretion and phagocytosis (Mewes *et al.*, 2009; Cortes *et al.*, 2012, 2014). I have shown that DrLITRs contain various tyrosine-based motifs and it is possible that these motifs may activate signalling pathways in a similar way as other receptor systems. It is also possible that they may signal using entirely novel pathways, especially in the case of DrLITR 1.1 and DrLITR 1.2, as the presence of ITAM and ITIM in the CYT region of an individual immunoregulatory receptor-type has yet to be documented. Overall, the tyrosine-based motifs show functional versatility but the role of these motifs in DrLITRs can only be answered with further investigation.

The PSI-BLAST results suggest that DrLITRs are distantly related to mammalian FcRLs and LRC-encoded molecules, such as CEACAMs, PSGs and SIGLECs. Various LITR-types contain multiple Ig domains that are related to the domains these distinct mammalian receptor families. This suggests that LITRs, LRC-encoded molecules and FcRLs were once encoded on the

same chromosome but over evolutionary history, they diversified and split into different chromosomal regions during speciation. FcRLs are mainly involved in regulating B-cell signalling (Davis, 2007; Wilson, Fuchs and Colonna, 2012). SIGLECs are involved in activating phagocytosis of sialylated microbes as well as regulating B-cell signalling (Crocker, Paulson and Varki, 2007; Chang and Nizet, 2014). CEACAMs are involved in cell-to-cell adhesion, while PSGs are mainly involved in modulating maternal-fetal immune responses (Gray-Owen and Blumberg, 2006; Moore and Dveksler, 2014). In humans, FcRLs are encoded on chromosome 1 while LRC-encoded molecules are encoded on chromosome 19 (Trowsdale, 2001; Barrow and Trowsdale, 2008). LITRs are distantly related to these various mammalian receptors but this does not mean that LITRs perform the same functions as these receptors *in vivo*. However, this distant relationship does suggest that these receptors once existed on an ancestral chromosome in a common ancestor but have diversified over the course of evolutionary history. It can be speculated that LITRs may act as CEACAMs during development and may be involved in cell migration (Kuespert, Pils and Hauck, 2006). They may also function as FcRLs in adulthood and mediate the regulation of innate immune responses (Davis, 2007; Wilson, Fuchs and Colonna, 2012). It is also possible that, regardless of age, that LITRs are only involved in host protection against various pathogenic challenges. The role of these receptors can only be speculated until further studies are performed to fully elucidate their immunoregulatory roles. According to my data, DrLITRs are ubiquitously expressed suggesting that these receptors may potentially play a role in every stage of zebrafish life.

In summary, the data presented in chapter IV shows the identification and molecular characterization of four LITR-types in zebrafish. I have identified all the structural characteristics

of the receptors and speculated some potential functions of DrLITRs *in vivo*. One of the biggest limitations to LITR research was that all functional capabilities were described *in vitro*. The first step to expanding LITR research *in vivo* is to identify these receptors in a new (i.e. different) teleost species. I have set the stage to further LITR research by establishing an *in vivo* model (i.e. zebrafish) in which four LITRs have been identified and their structural features annotated.

6.1.2 Expression analysis of select DrLITR-types

To elucidate the roles of LITRs, the question that needs to be answered is if LITRs are present, in the first place. Using my established qPCR assay, I showed that DrLITR 1.1, DrLITR 1.2, DrLITR 15.1 and DrLITR 23.1 are always expressed regardless of the age of the fish. There is no quantitative gene expression data for LITRs therefore I made predictions about their expression profiles based on what was observed for other innate immune markers. I predicted to observe differential expression patterns, as seen for cytokines and other innate immune receptors, such as TLRs and NITRs. According to my results, DrLITRs do show differential expression patterns as each receptor's profile differs from the rest. Additionally, most innate immune markers, including DrLITRs, are present from the time of birth. They may be present for protection or for some other developmental roles, but they do seem to be maternally sourced and continue to be sustained due to embryonic transcription. Maternal mRNA is passed down to the offspring while the embryo uses miR-430 to degrade maternal mRNA to facilitate its own transcription. miR-430 is detected at around 2 hpf and is one of the first transcripts to be transcribed by the embryo (Giraldez *et al.*, 2006; Heyn *et al.*, 2014). One of the main functions of this miRNA is to degrade the maternally sourced mRNA. It has been shown that when maternally sourced mRNA is not degraded, the accumulation of translated proteins

results in embryo mortality by day 5 (Giraldez *et al.*, 2005). Therefore, miR-430 is crucial for the survival of the fish early in development. The mother passes down RNA that may be important in embryo survival and may have some role in aiding embryogenesis. However, the embryo also contributes to its own survival via the functions of miR-430. In summary, the presence (or absence) of transcripts during development seem to be co-regulated by the mother and the embryo.

DrLITR 1.1, DrLITR 1.2 and DrLITR 23.1 have higher expression levels during early development while DrLITR 15.1 have lower levels. All of these receptors have different relative fold changes when compared to one another. In other words, they may follow similar trends but the actual quantified gene expression levels differ between receptors. These differences in expression levels may be indicative of the notion that each DrLITR may be involved in different processes during development and therefore, the fluctuations (i.e. upregulation and downregulation) may correlate to their specific roles. For instance, early developmental events include cell division and segmentation of organs (Kimmel *et al.*, 1995). It is possible that DrLITR 1.1, DrLITR 1.2 and DrLITR 23.1 may be involved in these key developmental events. Additionally, DrLITR 15.1 might be involved in the growth phase of the embryo which is characteristic of later developmental stages (Kimmel *et al.*, 1995). Alternatively, it is possible that the main function of DrLITRs is only for the protection of the embryo from pathogen infiltration and therefore, they are present from birth to ensure the survival of the fish. To conclude, DrLITRs are always present during ontogeny but it is unknown why they are present. Future studies are required to elucidate the molecular reasons behind the differences in their gene profiles.

Similar to what was seen in embryos, DrLITRs displayed variable gene expression levels when exposed to zymosan. Each DrLITR had its own unique upregulation/downregulation signature that also varied when compared to the expression of IL1β. DrLITR 1.1 was significantly upregulated at 12 hours post zymosan exposure while DrLITR 1.2 was significantly upregulated at 3, 6 and 12 hours. DrLITR 15.1 was unaffected at each time point while DrLITR 23.1 was downregulated during the 24 hour time point. The reasons behind these fluctuations could be differential leukocyte recruitment, efferocytosis and/or leukocyte altering receptor profiles on the surface of their cells. It is well known that macrophages are always migrating throughout the body and are recruited to the site of pathogen infiltration (Duque and Descoteaux, 2014). Additionally, it is known that LITRs are expressed on macrophages (Stafford *et al.*, 2006). Therefore, it is possible that the "upregulation" of DrLITR 1.1 and DrLITR 1.2 can be attributed to their presence on infiltrating macrophages. They may not directly be involved in zymosan induced inflammatory responses but their presence on innate immune cells directly contributes to their high expression levels. Efferocytosis is the process of engulfing host cells that have become apoptotic due to injury or infection (Green, Oguin and Martinez, 2016; Elliott, Koster and Murphy, 2017). This response requires coordination between two receptors for the successful engulfment of apoptotic cells in a process called "tethering and tickling". One receptor regulates the binding of the dying host cell to the surface of a phagocyte (i.e. "tethering" receptor) while another receptor mediates the engulfment process (i.e. "tickling" receptor). DrLITR 15.1 and DrLITR 23.1 were relatively unaffected by the zymosan exposure and this could mean that they may be "tethering" receptors during immune responses. They are needed for the successful engulfment of the target via "tickling" receptors but are not

necessarily upregulated. In contrast, it is possible that DrLITR 1.1 and DrLITR 1.2 are "tickling" receptors present on phagocytes and therefore, the increase of phagocytes during immune responses has a direct effect on the upregulation of these receptors. Additionally, the general trend suggests that DrLITR 15.1 and DrLITR 23.1 are downregulated during zymosan treatment when compared to PBS. An explanation for this could be the fact that leukocytes can alter their receptor profile to more efficiently deal with pathogen infiltration. For example, NK cells upregulate the surface expression of chemokine receptors under hypoxic conditions (Parodi *et al.*, 2018). It is possible that leukocytes downregulate the expression of DrLITR 15.1 and DrLITR 23.1 to upregulate the expression of other immune receptors such as chemokine receptors or even DrLITR 1.1 and DrLITR 1.2. These explanations are mere guesses based on what is seen in literature for other immune markers and they will continue to remain speculative until DrLITRs are further characterized.

6.2 FUTURE DIRECTIONS

6.2.1 Tissue-specific examination of DrLITRs

One of the caveats to my experiments is that I didn't analyze the tissue specific expression of DrLITRs. I extracted RNA from the entire viscera but it is unknown if one organ expresses more DrLITR transcripts than the others. For instance, IpLITRs are known to be expressed primarily by immune cells and in immune tissues (e.g. spleen, gill and kidney)(Stafford *et al.*, 2006). The question remains to be answered if the spleen, for example, in the viscera of the zebrafish expresses more transcript than the intestines, pancreas and liver. Additionally, if DrLITRs are highly expressed in lymphoid organs then it will further support the notion that LITRs are most likely involved in immune processes.

One way to analyze tissue-specific expression would be to utilise my developed qPCR assay. RNA would have to be extracted from various organs, such as muscle, kidney, spleen, pancreas, intestines, liver, heart and gills. After RNA extraction, cDNA can be synthesized and the qPCR primers reported for DrLITR 1.1, DrLITR 1.2, DrLITR 15.1 and DrLITR 23.1 in Chapter III can be used to create an expression profile for these receptors. An additional advantage to analyzing tissue-specific expression is to observe the presence of isoforms. It is possible that isolating individual organs may produce isoforms as the RNA extracted is highly specific to one organ. This may result in organ-specific isoforms being produced. In the viscera, this specificity is lost, and therefore, the potential detection of splice variants is also being lost. For example, it is possible that the spleen might be expressing isoforms but in the viscera, there is only one spleen so there is not enough spleen-specific RNA present and therefore, some isoforms might not be detected. Additionally, new research questions can be asked if various immune challenges would result in the production of isoforms in certain tissues. The production of splice variants can be indicative of the notion that certain LITRs are only needed for that immune challenge and this information may aid in the identification of LITR functions *in vivo*.

6.2.2 *In vitro* examination of DrLITRs

All previous LITR functional studies have been done *in vitro*. Although, *in vitro* studies cannot be extended into *in vivo* situations, a lot can be learned from the functional outputs of receptors in mammalian cell lines. Heterologous expression systems are an economically feasible, efficient and relatively quick way to characterize the functional capacities of receptors. Previous members of the Stafford lab have created and optimized the protocols for transfecting IpLITR transcripts into mammalian cell lines and therefore, these assays can be used to

transfect DrLITR transcripts into immune (i.e. RBL-2H3) and non-immune (i.e. AD293) cell lines (Mewes *et al.*, 2009; Cortes *et al.*, 2012; Zwozdesky *et al.*, 2017). The CYT regions of DrLITRs have similarities and differences with IpLITRs. For instance, DrLITR 15.1 has the same tyrosine-based motifs as IpLITR 1.1b. DrLITR 15.1 has two overlapping ITIMs and one ITSM while IpLITR 1.1b has overlapping ITIM and ITSM and an additional ITIM. DrLITR 23.1 is similar to IpLITR 2.6b as they both don't have any recognizable tyrosine-based motifs but do consist of a positively-charged TM segment. DrLITR 1.1 and DrLITR 1.2, on the other hand, have unique CYT regions with the presence of both ITIM and ITAM which has never been observed in IpLITRs or any other receptor system defined in literature. The presence of both stimulatory and inhibitory motifs does suggest that these receptors might show signalling versatility. They may be involved in immune system activation and inhibition. This can be tested very easily using *in vitro* systems through the use of phagocytosis assays, optimized in the Stafford lab (Cortes *et al.*, 2014; Lillico *et al.*, 2015). The question that remains to be answered is if DrLITR 15.1 and DrLITR 23.1 would also mediate immune responses in a similar way to what was observed in IpLITR 1.1b and IpLITR 2.6b, respectively. The ability of fish receptors to mediate immune responses in mammalian cell lines further supports the idea of conserved cellular machinery throughout evolution. This highlights the importance of studying non-mammalian models in order to expand our understanding of mammalian signalling capacities..

6.3 FINAL CONCLUSIONS

Since innate immunity is highly conserved, non-mammalian models can be used to examine and broaden our understanding of innate immune processes. One main conclusion that can be made from my thesis findings is that the DrLITR transcripts are present at varying

levels throughout ontogeny and adulthood. Based on my results and previous knowledge about LITRs, only speculations can be made about the role of these receptors. I have set the stage by establishing an *in vivo* model in which four LITRs with unique structural compositions have been identified and I have also reported their quantified gene expression. Additionally, I have also created receptor structures based on cDNA and have fully identified the 3' end of the protein sequence ensuring the unique CYT regions are complete. I have even identified receptor schematic differences (i.e. DrLITR 15.1 and DrLITR 23.1) that was found between the reference genome and the expressed cDNA. In the future, these receptors can be analyzed further to identify some potential functions. If researchers, in the future, choose to identify additional LITR-types or quantify gene expression in fish, then my established protocols can be utilised and optimized to further LITR research. In any case, an *in vivo* model is an extremely useful tool as the results of experiments can be directly applied to how an organism might respond under actual environmental conditions. Therefore, continuing LITR research in zebrafish (i.e. *in vivo*) is key to understanding the vital functions of LITRs during development and adulthood.

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