ABSTRACT

YANG, JIBING. Immune-related Lectin-Like Receptors in Zebrafish Innate Immunity. (Under the direction of Dr. Jeffrey A. Yoder.)

The Superfamily of lectin receptors is composed of proteins that recognize diverse ligands including carbohydrates present on bacteria and fungi or stress-related and viral proteins. Natural killer (NK) cells are a subset of lymphocytes that can recognize and directly kill virally infected or transformed cells. This recognition is mediated through their cell surface receptors including the Group V C-type lectin-like domain (CTLD) receptors (also known as NK cell receptors or NKRs). NKRs in different mammalian species have been widely studied in the past two decades. However, in lower vertebrates, the knowledge of NKRs is limited to genomic sequence or computational structure analysis in several bony fish species. It would be valuable to identify orthologs of NKRs in non-mammalian vertebrates and explore the evolutionary function of these receptors. In this regard, our previous studies have characterized a family of immune-related, lectin-like receptors (ILLRs) in zebrafish that are structurally similar to Group II CTLD receptors yet contain intracellular signaling domains similar to Group V NKRs. In order to better understand the role of ILLRs in zebrafish innate immunity, it is necessary to develop a way to enable identification, purification, characterization and functional analysis of ILLR expressing cells.

Chapter I is an introduction to discuss C-type lectin receptors and zebrafish innate immunity providing an ideal background for these studies. Chapter II investigates the expression patterns of ILLRs at the cellular level. RNA *in situ* hybridization from wild type zebrafish and flow cytometric analysis of cells from transgenic fish did not successfully detect ILLR

expressing cells due to low levels of expression. Therefore, the goal of Chapter III was to develop a method to boost ILLR expression in *vivo* and facilitate the identification and purification of ILLR expressing cells. Chapter IV applied the immune-challenged model developed in Chapter III to transgenic fish to identify ILLR expressing cells. Chapter V explores additional ILLR genes in zebrafish on Chromosome 19. Current results indicate that *illrL* gene expression increases dramatically upon pathogen infection, suggesting that C-type lectin receptors in zebrafish may be conserved as the host-pathogen interaction molecules as shown in mammals and may play a critical role in zebrafish innate immunity.

Immune-related Lectin-Like Receptors in Zebrafish Innate Immunity

by
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BIOGRAPHY

Jibing Yang was born in Hubei, China, in 1978. He received his bachelor degree in Veterinary Medicine from China Agricultural University in 2001. After graduation, he did an internship in small animal medicine at the Veterinary Teaching Hospital at China Agricultural University. In 2005, he received his Master degree in Biomedical and Veterinary Sciences under the direction of Dr. Sharon Witonsky from Virginia-Maryland Regional College of Veterinary Medicine at Virginia Tech. He enrolled in the PhD program in immunology at North Carolina State University in Fall 2005 and joined Dr. Jeffrey Yoder's lab to study immune-related lectin-like receptors in zebrafish innate immunity.

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LIST OF ABBREVIATIONS

ADCC - antibody dependent cellular cytotoxicity

AP - Alkaline Phosphatase

BAC - Bacterial Artificial Chromosomes

BCIP - 5-Bromo-4-Chloro-3'-Indolyphosphate p-Toluidine

C/EBPβ - CAAT enhancer-binding protein β

CLRs - C-type lectin receptors

CRDs - carbohydrate recognition domains

CTLDs - C-type lectin-like domains

DEPC - diethylpyrocarbonate

DIG - digoxigenin

DNA - Deoxyribonucleic Acid

dNTP - deoxynucleoside triphosphates

ECP - Extracellular products

FACS - Fluorescence Activated Cell Sorting

FBS - Fetal bovine serum

FITC - Fluorescein Isothiocyanate

GFP - green fluorescent protein

HPI - hours post infection

IL-1 - interleukin-1

ITAM - immunoreceptor tyrosine-based activating motifs

ITIM - immunoreceptor tyrosine-based inhibitory motifs

ILLR - immune-related, lectin-like receptor

Ig - immunoglobulin

KIR - killer cell Ig-like receptors

LIR - leukocyte Ig-like receptors

LN - Lymph Nodes

LPS - lipopolysaccharides

MHC 1 - major histocompatibility complex I

mAb - monoclonal antibody

NBT - Nitro-Blue Tetrazolium Chloride

NITRs - novel immune-type receptors

NK - Natural killer

NKRs - NK cell receptors

ORF - Open Reading Frame

PAMP - pathogen associated molecular pattern

PBMC - Peripheral Blood Mononuclear Cells

PBS - Phosphate buffered saline

PCR - Polymerase Chain Reaction

PFA - paraformaldehyde

qRT-PCR - Quantitative reverse-transcriptase PCR

RACE - Rapid amplification of complementary DNA ends

RAE-1 - retinoic acid early inducible-1

RAET-1 - retinoic acid early inducible-1 like transcripts

RNA - Ribonucleic Acid

RISH - RNA *in situ* hybridization

RT - Reverse Transcriptase

SHRV - *Snakehead rhabdovirus*

S. iniae - *Streptococcus iniae*

WT - wild type

TFBS - transcription factor binding sites

Tg - transgenic

ULBP - UL-16 binding proteins

CHAPTER 1. LITERATURE REVIEW

1.1 Introduction

C-type lectins are typically defined as proteins which bind carbohydrates through calcium-dependent carbohydrate recognition domains (CRDs). The first C-type lectins were described in 1860 as snake venom lectins due to the agglutinating activity (1). With the discovery of more C-type lectins, comparison of the CRD sequences from them revealed some conserved residue motifs which allowed more proteins with these motifs to be found (2-5). As the numbers of proteins in this family grew, researchers found that some of them did not contain authentic CRDs. To resolve these differences, the more general term C-type lectin-like domains (CTLDs) was introduced to refer to this family (6, 7). Currently, based on the domain architecture, the vertebrate CTLDs family has been classified into 17 groups (8). The simplified structures of the 17 groups of the CTLD protein family are shown in Figure 1. Numerous mammalian proteins which possess CTLDs are well known to play important roles in the immune system by recognizing pathogen-associated carbohydrates that are found exclusively on pathogens, or that are inaccessible in host cells (7, 9-11). However, many members of the CTLD family do not bind carbohydrates such as the group V NK cell receptors which are non-classical C-type lectin receptors. They recognize protein ligands independent of calcium and include the multigene families of Ly49 in mice and NKG2 in both mice and human which have been characterized based on their function in NK cells (12). In order to clarify the ambiguousness in ligand recognition, we use CLRs to refer to C-type lectin/lectin-like receptors in the introduction. Table I summarizes the important CLRs involved in the immune responses. Protein-carbohydrate interactions in the immune system

mainly mediate two functions: cell-cell interaction and pathogen recognition (10). The soluble collectins mediate foreign molecule neutralization though activation of the complement pathway (13). The mannose-binding receptors on the macrophage cell surface recognize pathogen associated molecular patterns (PAMPs) which lead to phagocytosis of microorganisms. The selectins mediate leukocyte trafficking across the endothelium. CLRs which bind ligands other than carbohydrates may interact with protein targets.

Figure 1. Overview of simplified structure for each CTLDs subfamily. Group numbers are indicated besides the domain charts. Group I – lecticans, II – the ASGR group, III – collectins, IV – selectins, V – NK receptors, VI – the macrophage mannose receptor group, VII – REG proteins, VIII – the chondrolectin group, IX – the tetranectin group, X – polycystin 1, XI – attractin, XII – EMBP, XIII – DGCR2, XIV – the thrombomodulin group, XV – Bimlec, XVI –SEEC, XVII CBCP. Transmembrane CTLDs subfamilies include Group II, IV, V, VI, VIII, XV, X, XI, XIII, XIV. Figure adapted fromZelensky and Gready, 2005 (8).

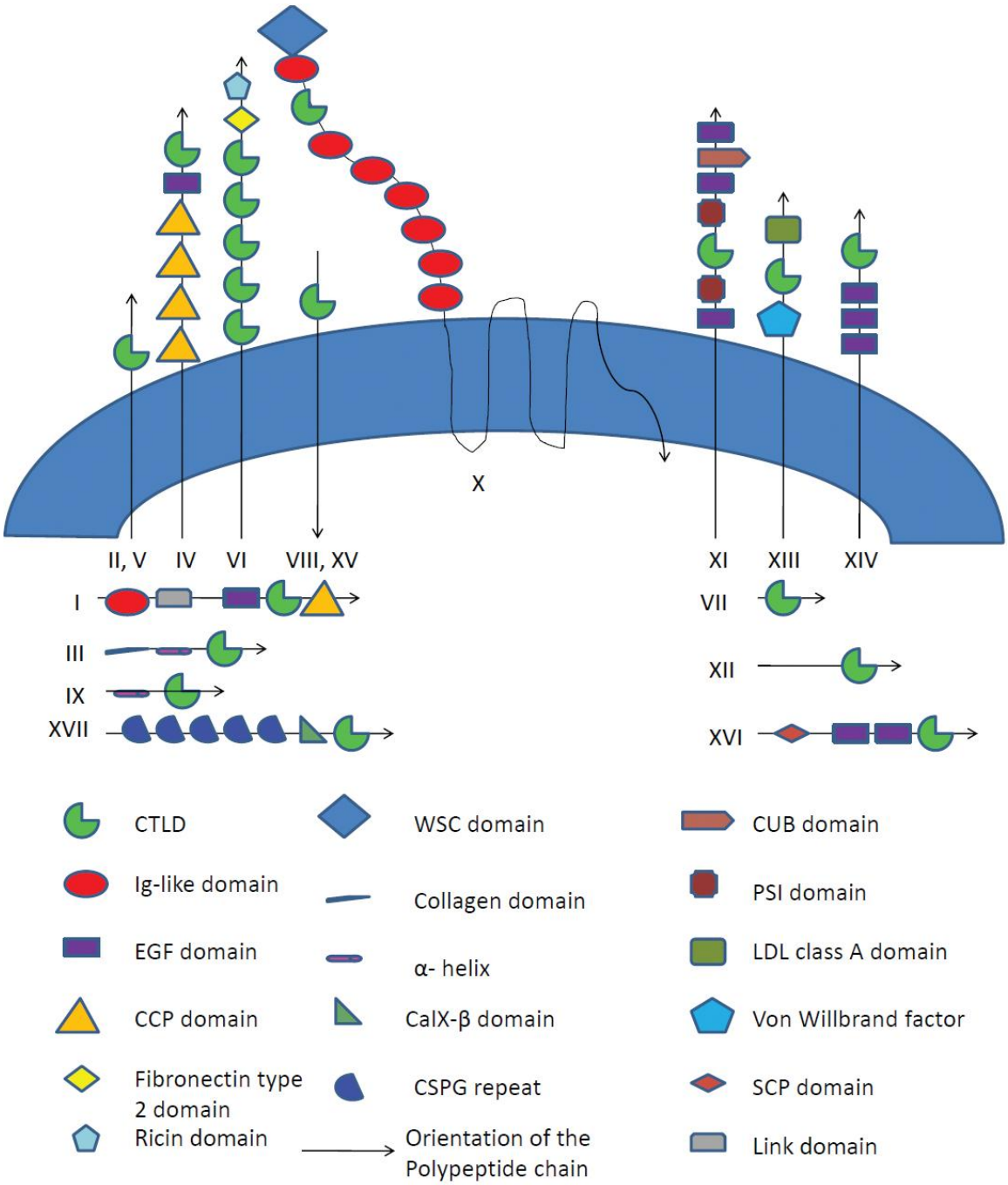

II, V
IV
VI
VIII, XV
X
XI
XIII
XIV
I
III
IX
XVII
VII
XII
XVI
CTLD
Ig-like domain
EGF domain
CCP domain
Fibronectin type 2 domain
Ricin domain
WSC domain
Collagen domain
α- helix
CalX-β domain
CSPG repeat
Orientation of the Polypeptide chain
CUB domain
PSI domain
LDL class A domain
Von Willbrand factor
SCP domain
Link domain

Table 1. A summary of some important CLRs in immunity

Receptors	Group	Ligand specificity	Expression	Function	Refernces
DC-SIGN	II	ICAM-2, ICAM-3, HIV	DCs, MΦ	Cell adhesion, pathogen recognition	(11)
Ficolin	III	Carbohydrates	Liver, uterus	Pathogen recognition	(14, 15)
Collectins	III	Carbohydrates	Soluble	Pathogen recognition, complement activation	(14, 16)
MMR	IV	Mannose, Fucose	DCs, MΦ,langerhans cells, lymphatic endothelium	Cell adhesion, pathogen recognition	(10)
Selectins	IV	Carbohydrate, glycoproteins	Endothelium, leukocytes, platelets	Cell adhesion	(8, 10)
Dectin-1	V	β -glucan	DCs, MΦ, neutrophils	Anti-fungi immunity	(17, 18)
CLEC (Dectin-1 cluster)	V	Endogenous and exogenous ligands	Variable cells	Immunity and hemeostasis	(17)
Mincle	II	Spliceosome-associated protein 130	MΦ, neutrophils	Recognize necrotic cells	(19)
NK cell receptors (NKG2, Ly49)	V	Carbohydrates, proteins	NK cells, antigen presenting cells, T cells	Cell adhesion, antigen uptake	(8, 10)

NK cells are a distinct subset of lymphoid cells that have immune function in surveillance of virus-infected cells or neoplasia (20). This is accomplished after the NK cell surface receptors (NKRs) bind their ligands. In general, NK cells express a repertoire of inhibitory receptors that recognize major histocompatibility complex I (MHC I) whereas different activating receptors have been shown to bind variants of MHC I or virally encoded or stress-induced proteins that are structurally similar to MHC I (21). In the case of NKRs, little is known about their structure, ligands and function below the phylogenetic levels of mammals, and even inside mammals (22, 23). Analysis of the *Fugu rubripes* genome failed to identify any Group V NKRs (24). To better understand the evolutionary role and divergence of NKRs in innate immunity it is necessary to study their structure and function in non-mammalian species. Recently, investigators have characterized a family of immune-related lectin-like receptors (ILLRs) in zebrafish that are structurally similar to Group II CLRs yet contain intracellular domains similar to Group V NKRs (25). The zebrafish is increasingly being used for the discovery of human disease genes as well as for studying the immune system and host defense (22, 26, 27). Zebrafish share many orthologous genes with human and mouse, which makes it an excellent model system to understand phylogenetically conserved, as well as divergent, mechanisms of pathogen recognition and evolution of innate immunity.

The current study was conducted to enhance our understanding of the roles of CLRs governing innate immunity against infection in zebrafish. Some cutting-edge technologies in zebrafish research were utilized to characterize ILLRs at the molecular and cellular level.

The focus of the Introduction is to highlight important fundamental concepts and background knowledge related to this research.

1.2 Zebrafish as a model in immunology

Zebrafish was first introduced as an animal model into the immunology research community a decade ago when biologists utilized the zebrafish to study the development of the immune system (28). Zebrafish has been established as an excellent model for vertebrate development because of its inherent advantages. These include ease of genetic manipulation and transgenesis, short generation time, transparent embryos, small size, and easy maintenance, which helps make it an outstanding model system for investigating organ development (such as eye, kidney, heart), cancer biology, neurobiology, toxicology, and drug discovery (29-36). Although the mouse is the most common model organism in immunology research, mice are expensive to maintain, develop in utero making embryo manipulations challenging, and are limited to small-scale genetic studies. Zebrafish nicely complement these deficiencies in mice. Lymphoid tissues can be studied in zebrafish at earlier stages of immune system development (22). For example, T lymphocytes can be identified in the thymus by 4 days post fertilization (dpf) in *lck-GFP* T-cell lineage transgenic fish (37). RNA *in situ* hybridization studies show that T lymphocytes appear in the peripheral organs at 9 dpf which is consistent with the presence of mature T cells in the medulla of the thymus (38). Zebrafish are also particularly useful for the examination of how B lymphocytes develop and differentiate in different tissues. Both *Ig* μ and *rag1* genes are detected at 10 dpf in the

pancreas which may be equivalent to the white pulp of mouse spleen (39). Zebrafish also possesses a robust innate immune system. Highly polymorphic novel immune-type receptors (NITRs) which belong to the immunoglobulin superfamily (IgSF) and share similar structures with mammalian NK cell receptors (KIRs) have been characterized in zebrafish (40). Of the 44 NITRs, Nitr9 is the only activating receptor associated with Dap 12 to activate downstream signaling pathways similar to activating human KIRs (41). In addition, conserved markers for zebrafish neutrophils (*mpo*) and monocytes (*L-plastin*) have been identified (42, 43). Several transgenic zebrafish lines such as zMPO:GFP and lysC:EGFP have been developed to fluorescently tag neutrophils and monocytes permitting studies which observe inflammation *in vivo* (44, 45). Taken together, the strong conservation in immunity between zebrafish and mammals supports it as a feasible model for defining the molecular mechanism of immune system development and human diseases.

Recently zebrafish has also become an excellent model to study host-pathogen interactions (46-48). Several different microorganisms have been used to infect either zebrafish embryos or adults to build alternative model systems for studying the immune response against infection (49-51). B and T lymphocytes do not become mature until 4 to 6 weeks post fertilization, which makes zebrafish embryos a useful model to determine the role of innate immune response against different pathogens (52). Functional knock-down of MyD88 decreased the zebrafish embryo's ability to clear *Salmonella enterica* (53). Exposure to bacterial lipopolysaccharide (LPS) and the fish pathogen *Edwardsiella tarda* induced expression of pro-inflammatory cytokines IL-1β and TNF-α (54, 55). These data validate the

zebrafish embryo as a useful model for study of the vertebrate innate immune system. Adult zebrafish can also be infected by similar pathogens as mammals. For example, intramuscular injection of *S. iniae* results in infection of zebrafish (56, 57). These infection models may eventually allow investigators to elucidate host-pathogen interactions at the molecular level for human diseases.

1.3 CLRs on myeloid cells

CLRs present on myeloid cells belong to groups of pattern recognition receptors (PRR). Many CLRs are traditionally thought to be NK cell receptors since they possess activating or inhibitory intracellular signaling motifs, suggesting that they may positively or negatively regulate the function of myeloid cells (58). CLRs identified on myeloid cells recognize structurally diverse ligands and have a variety of immune and homoeostatic functions compared to their NK counterparts. They may belong to Groups II, IV, and V (Table 1). Here, I review two of these CLRs in order to explore our current understanding of their ligands and functions. The first one is CLEC2 belonging to Group V Dectin-1 cluster of CLRs, which includes MICL, CLEC2, CLEC12B, CLEC9A, CLEC-1, Dectin-1 and LOX-1 (17). CLEC2 contains an activating motif and is expressed on myeloid cells and platelets (59, 60). Upon binding to its ligand the snake venom component rhodocytin, CLEC2 is phosphorylated at its intracellular activating domain, which recruits Syk kinase and activates the downstream phosphorylation pathway including PLCγ2 (60). Although, CLEC2 shares a similar activating signal pathway as NKG2D and Ly49, CLEC2 does not bind to the same ligands

such as MHC class I (59). The physiological function and endogenous ligands of CLEC2 still remain unknown. The second CLR is Ly49q, a group II CLR. It contains a single CRD in its extracellular region and an inhibitory motif in its cytoplasmic domain. Unlike other members of the Ly49 family which are expressed exclusively on NK and T cells, Ly49 is expressed on myeloid precursors in mouse bone marrow cells, monocytes, and subsets of dendritic cells (61, 62). The maturational status of DC affects Ly49q expression, and IFN-α can significantly enhance its expression on myeloid DC (61). Ly49q may recognize MHC class I molecule H-2K(b) on activated B cells to induce the maturation of mouse plasmacytoid DC (pDC) (63). A recent study reports that Ly49q is important for TLR9-mediated type I IFN production by pDCs, suggesting that Ly49 may have an endogenous role in linking the innate with the adaptive immune response (64). In addition to pathogen recognition, CLRs on myeloid cells also participate in microbe phagocytosis, induction of innate response genes, and binding to endogenous ligands as self-surveilence (65, 66). A deeper understanding of how CLRs function on myeloid cells may reveal some unknown mechanisms of how innate and adaptive immunity are regulated.

1.4 Natural Killer cells and their receptors

1.4.1 Identification of NK cells

NK cells were originally described in 1975 as a subset of lymphocytes capable of killing tumor cells without previous sensitization (67, 68). In 1981, Timonen et al. defined NK cells

as large granular lymphocytes, but activated cytotoxic T cells also displayed this morphology (69, 70). Since NK cells do not express TCR/CD3 complexes and cannot be classified as T or B lymphocytes, they were called "null" cells at the time based on the lack of T or B cell surface markers. Later, Lanier et al. identified the first NK cell surface marker CD16 on a subset of human peripheral blood lymphocytes (71). We now know that CD16 is a Fc receptor and also a NK cell activating receptor which mediates antibody dependent cellular cytotoxicity (ADCC) function. Several surface markers or receptors have been elucidated on NK cells, but mice and human do not share some NK markers. Although NKp46 is a member of the natural cytotoxicity receptor family and has been defined across species (72), it also appears on a subset of human T lymphocytes (73). In general, the NK1.1 is a useful surface marker for mouse NK cells in C57/BL strains (74). Administration of the monoclonal antibody (mAb) PK 136 against NK1.1 can selectively deplete NK cells (75). Other cell surface markers such as CD11b and CD27 have also been used to identify NK cell subsets in mice (76). CD11b also expressed on monocytes, macrophages, and DC. In man, two subsets of NK cells have been described based on their function. $CD56^{dim}/CD16^{+}$ NK cells represent 90% of peripheral blood-derived and spleen-derived NK cells and are associated with cytotoxicity while $CD56^{bright}/CD16^{-}$ NK cells in lymph nodes and tonsils produce higher levels of cytokines (77). Therefore, NK cells are very distinct from B and T lymphocytes in cell surface markers. Note that the above mentioned markers are associated with natural killing capacity, but are not necessary for target recognition discussed next.

1.4.2 NK cell receptors

NKRs are germline-encoded and not unique to individual NK cells (78). This feature differentiates NKRs from TCRs which undergo somatic recombination to generate antigen-specific receptors. NK cells function normally in mice with defects in recombination such as *scid* mice or *Rag1* or *Rag2* knock-out mice (74, 79, 80). NKRs can be classified into two structural families: immunoglobulin (Ig)-like receptors and lectin-like receptors. Each family includes both inhibitory and activating receptors. Ig-like receptors include killer cell Ig-like receptors (KIR), Ig-like natural cytotoxicity receptors (NCR), and leukocyte Ig-like receptors (LIR). First, the KIRs are a multigene family and are often highly polymorphic. There are 16 different KIR genes that are encoded on human chromosome 19q13.4 (81). KIRs generally recognize HLA class I ligands. Second, Ig-like natural cytotoxicity receptors (NCR: NKp30, Nkp44, Nkp46) associate with different adaptor proteins (CD3ε, FcγRIg or DAP12) bearing cytoplasm immunoreceptor tyrosine-based activating motif (ITAM) to deliver an activating signal to NK cells (82). Although NCRs were first identified in tumor cells, the cellular ligands recognized by NCRs are still unknown. Finally, LIR1 (ILT2/CD85j) belongs to the leukocyte Ig-like receptor family which is located on human chromosome 19 (q13.2-q13.4) linked to the FcαR and KIR genes (83). LIR1 broadly recognizes HLA class I molecules to suppress NK cell responses via its cytoplasmic immunoreceptor tyrosine-based inhibitory motifs (ITIM) domain. The LIR family includes two activating receptors LIR6 and LIR7.

Lectin-like NKR families include Ly49 in mice and CD94/NKG2 in humans and mice. The mouse Ly49 family comprises at least 23 members (Ly49A through W), which are type II transmembrane glycoproteins with each chain composed of an extracellular CTLD, a stem region, and a transmembrane domain (84). All members of the Ly49 family are disulfide-linked homodimers through paired cysteine residues in the extracellular stem region (85). Activation of Ly49D results in the production of cytokines and chemokines, in addition to lysis of virally infected cells (86). Human NKG2D is a lectin-like activating receptor expressed on NK cells and T cells. It recognizes ligands such as MICA/B, a family of proteins called "UL-16 binding proteins" (ULBP), or retinoic acid early inducible-1 (RAE-1) like transcripts (RAET-1) on infected, stressed, and transformed cells (87, 88). CD94/NKG2C shares the same ligand HLA-E as the inhibitory NKR CD94/NKG2A (89).

1.4.3 Function through ITAM or ITIM

NK cells, as a component of innate immunity, are generally thought to be the first line of defense against viral infections and tumor cells. The mechanism of NK cell killing includes two different pathways: direct cellular cytotoxicity and the production of cytokines such as IFN-γ. NK cell cytotoxic function is carefully controlled by a balance between their activating and inhibitory receptors. The "missing-self" hypothesis explains how NK cells recognize and kill target cells (90). Many inhibitory NKRs recognize HLA class I molecules as "self" to inhibit target cell killing. All the inhibitory receptors share an amino-acid sequence motif in their cytoplasmic tail named ITIM. After inhibitory receptors interact with

their ligand, their ITIM motif becomes phosphorylated by enzymes of the Src family of kinases, allowing them to recruit other enzymes such as the phosphotyrosine phosphatases SHP-1 and SHP-2, or the inositol-phosphatase called SHIP (91). These phosphatases decrease the activation of molecules involved in cell signaling. Activating NKRs do not have an ITIM motif in their cytoplasmic tail and associate with adaptor proteins containing an ITAM or an YxxM motif in their cytoplasmic domain (92). Ligand binding by an activating receptor leads to tyrosine phosphorylation of the coupled adaptor protein which recruits and activates protein tyrosine kinases such as Syk/ZAP70 or PI3 (92).

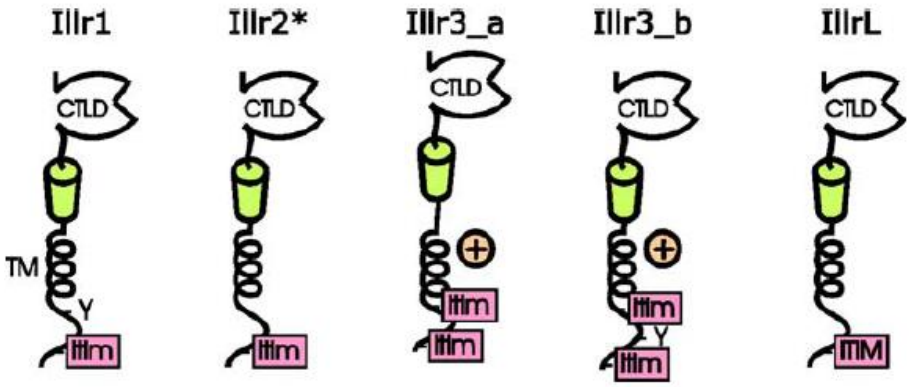
1.5 Lectin-like receptors in zebrafish

Innate immunity mediated through CLRs has stimulated interest in the past years. As discussed before, much of the available knowledge about CLRs is derived from studies in mammals. In fish species, C-type lectins have been cloned in rainbow trout (*Oncorhynchus mykiss*), carp (*Cyprinus carpio*), eel (*Anguilla japonica*), salmon (*Salmo salar*), and Japanese flounder (*Paralichthys olivaceus*) (93-97). Many lectins associated with eggs of several teleosts have also been isolated and partially characterized (98, 99). A cluster of C-type lectin NKR-like loci has been identified in bony fish (*oreochromis niloticus* and *bidochromis chilotes*) using a cDNA probe from killer cell-like receptor genes (100, 101). This series of novel research raises a new question for immunologists studying fish. Is it possible to identify NK cells in bony fish with this CLRs gene family *in vivo*? Recently, our investigators have identified and characterized a multigene family of ILLRs from zebrafish

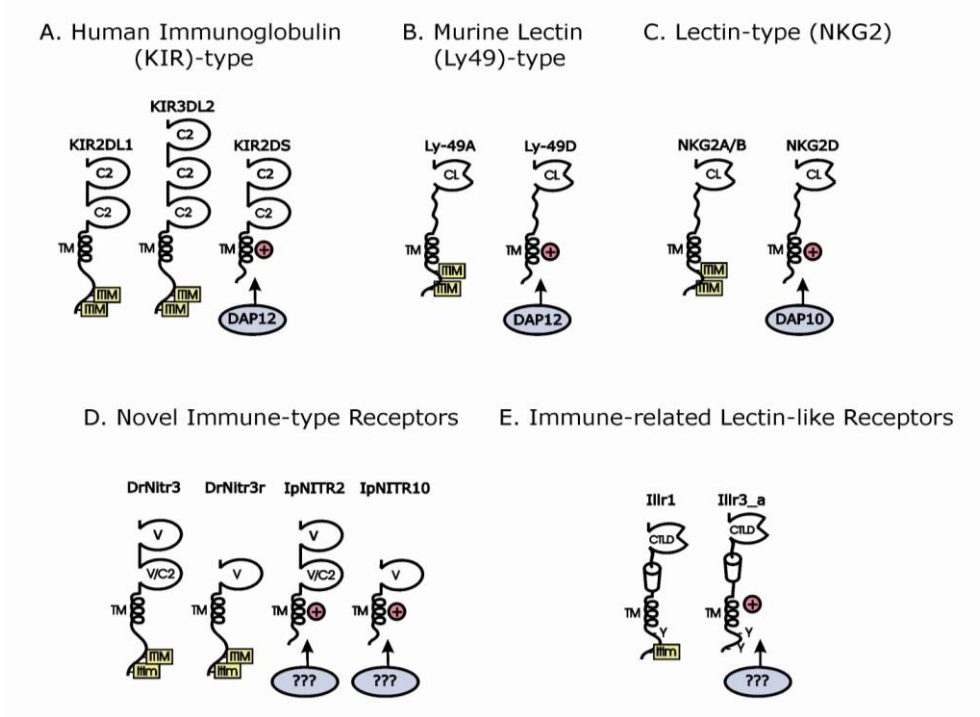
(25). This family consists of 4 closely related genes: *illr1*, *illr2*, and *illr3* are present as a gene cluster on chromosome 19 whereas *illrL* (also called *illr4* in Chapter 3) is encoded at a distal locus on the same chromosome. As *illr2* transcripts have not been identified, it is unknown if *illr2* is a pseudogene. *Illr3* has two alternatively spliced variants named *illr3_a* and *illr3_b*. All the ILLRs encode type II transmembrane receptors with a single extracellular CTLD and share structural features with both Group II and V CTLD receptors (Figure 2). Phylogenetic analysis of CTLD of ILLRs to other CLRs indicates that they are similar to mammalian Group II receptors, but belongs to a distinct subgroup (25). Examination of the cytoplasmic signaling motifs of ILLRs reveals that IllrL contains a consensus cytoplasmatic ITIM and that Illr1, Illr2, and Illr3 have ITIM-like sequences named as itim, suggesting that they may deliver an inhibitory signaling pathway upon ligand recognition (25, 102). Although, Illr3 encodes putative itim sequences and may play an inhibitory role, Illr3 also possesses a positive charged residue (Arg) within the transmembrane domain, a typical feature of activating NKRs (103). This indicates that it may function in dual roles as an activating or inhibitory receptor depending on the ligands. RT-PCR analysis shows that the inhibitory *illr1* is expressed in lymphoid cells, *illrL* is expressed in myeloid lineage, and *illr3* is expressed in both lymphoid and myeloid cells (25). This further suggests that *illr1* and *illr3* are good candidate markers for zebrafish NKRs. Although *illr3* is expressed in both lymphoid and myeloid cells, it does not rule out this possibility. Some mammalian NKRs are also expressed in both hematopoietic lineages (Table I). Collectively, these observations and results suggest that ILLRs could be one type of NK cell CLRs in zebrafish.

Figure 2. Zebrafish ILLRs are structurally related to natural killer cell receptors. 1A shows the predicted protein structures of each ILLR. ILLRs are located on chromosome 19, and contain an extracellular CTLD, a transmembrane domain, and an intracellular ITIM or ITIM-like motif (*itim*). The single activating gene *illr3* consists of a positive charged residue (Arg) in its transmemebrane domain. *Illr3* has two alternatively spliced variants named as *illr3_a* and *illr3_b*. 1B structurally compares ILLRs with human KIR, mouse Ly49, NKG2, and zebrafish NITRs. Figure courtesy of J.A. Yoder.

1A.


Illr1
Illr2*
Illr3_a
Illr3_b
IllrL
CTLD
TM
Y
ITIM

1B.


A. Human Immunoglobulin (KIR)-type
B. Murine Lectin (Ly49)-type
C. Lectin-type (NKG2)
KIR3DL2
KIR2DL1
KIR2DS
C2
TM
ITIM
DAP12
Ly-49A
Ly-49D
CL
NKG2A/B
NKG2D
DAP10
D. Novel Immune-type Receptors
E. Immune-related Lectin-like Receptors
DrNitr3
DrNitr3r
IpNITR2
IpNITR10
V
V/C2
???
Illr1
Illr3_a
CTLD
Y

1.6 Conclusion

The superfamily of CLRs comprises a large group of extracellular Metazoan proteins which utilize CTLDs to recognize diverse ligands. Some of them are important components of innate immunity in mammals. NK cells provide the first line of defense against viral infection and transformed cells. Ly49 in mice and NKG2 represent Group V CTLD NKRs in mammals. Many genes encoding putative NK cell CLRs have been described in bony fish. However, none of them have been identified exclusively on NK cells. This leads us to use some cutting-edge techniques such as RNA *in situ* hybridization and transgenic fish lines to examine ILLR expression. Understanding the cellular expression pattern and function of ILLRs will enable us to further characterize NK cells in vertebrates. Our hypothesis is that ILLRs play an important role in zebrafish innate immune response in either recognizing pathogen-associated carbohydrates or mediating cytotoxicity to virally infected or transformed cells. More specifically for each ILLR gene:

1. *Illr1* is an inhibitory receptor expressed on NK cells and cytotoxic T cells.
2. *Illr3* is an activating transmembrane receptor and expressed on NK cells, cytotoxic T cells, and monocytes. Cells expressing *illr3* are cytotoxic.
3. *IllrL* is expressed on neutrophils or monocytes and could initiate immune responses following the recognition of pathogen-associated carbohydrates.

The following four chapters of this dissertation contain descriptions of studies designed to examine the following four four specific aims: Chapter II, evaluation of the cellular

expression pattern of ILLRs in healthy fish; Chapter III, characterization of ILLR expression levels after immune challenges; Chapter IV, investigation of *illrL* expression in transgenic zebrafish line (*illrL*^{2k}*:mCherry*); Chapter V, exploration of additional ILLR genes in zebrafish.

1.7 References

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CHAPTER 2. IDENTIFICATION OF ILLR CELLULAR EXPRESSION PATTERN IN ZEBRAFISH

Abstract

The multigene family of immune-related lectin-like receptors (ILLRs) encodes Group II C-type lectin receptors that possess inhibitory and/or activating peptide motifs typical of Group V C-type lectin NK receptors. A previous study demonstrated that the ILLR genes are differentially expressed in lymphoid and myeloid hematopoietic lineages, and share structural and functional characteristics with mammalian NKRs, suggesting that the ILLRs play important roles in zebrafish immunity. With the long-term goal to better understand the role(s) of ILLRs in zebrafish immunity, two strategies were employed to characterize ILLR expression patterns and identify ILLR expressing cells: 1. RNA *in situ* hybridization and 2. transgenic zebrafish lines in which putative ILLR promoters drive expression of a fluorescent reporter protein. RNA *in situ* hybridization studies indicate the low levels of ILLR expression resulting in the inability to define ILLR cellular expression patterns by histological assessment on either paraffin-embedded or frozen sections. A similar conclusion was drawn for the transgenic zebrafish. For example, flow cytometric analysis on *illr1*^{10k}*:EGFP* transgenic zebrafish kidney cells revealed a very low percentage of GFP positive kidney cells (0.47% in transgenic zebrafish vs 0.42% in wild type fish). In summary, our study shows that the ILLR genes are expressed at remarkably low levels which make it difficult to directly identify them in healthy fish.

Introduction

The C-type lectin protein superfamily includes a large group of lectin-like receptors in the animal kingdom. The typical characteristic feature of C-type lectin proteins is the presence of one or more C-type lectin-like domain (CTLD) which is a cysteine residue rich domain (1). These proteins have been classified into 17 groups (Group I, II, III,…XVII) based on the structural and functional features of the CTLDs (2). The classical C-type lectins are the largest and most diverse lectin family groups that recognize carbohydrate ligands in a calcium-dependent manner via their carbohydrate-recognition domains (CRDs) (3). The structure, function, and ligands of many of the classical C-type lectins have been well studied. Many Group II CTLDs proteins belong to classical C-type lectins, and contain a short cytoplasmic tail, transmembrane domain, an extracellular stalk region, and CRDs (2). However, non-classical C-type lectin receptors bind carbohydrates independent of calcium (4, 5). Many Group V CTLD proteins belong to this non-classical C-type lectin group and have evolved to recognize protein ligands, such as MHC class I (or related) molecules (6, 7). In mammals, numerous C-type lectins play important roles in immunity as mediators of cellular adhesion and as pathogen recognition receptors (8).

Recently, a few CLRs in bony fish have been cloned in rainbow trout, Japanese flounder, salmon, carp, and zebrafish (9-13). More importantly, Sato et al. described the first NK receptor with a CTLD in teleostean fish, *Paralabidochromis chilotes* (14). The multigene family of zebrafish ILLRs encodes Group II CTLD receptors which possess inhibitory and/or

activating signaling motifs typical of Group V CTLD (NK) receptors. The ILLR genes are differentially expressed in lymphoid and myeloid hematopoietic lineages, and share structural and functional characteristics with mammalian NKRs (11). Little is known about the cellular and functional features of ILLRs. The goal of this study was to identify ILLRs cellular expression pattern in zebrafish. Recently, zebrafish has gained prominence as a model system for immunological analyses with many advantages compared to mammalian speces including the ease of producing transgenic lines (Chapter I). Transgenic zebrafish expressing GFP under the control of gene-specific promoters allow us to track fluorescent cells expressing genes of interest. This technique has been extensively applied to studying T cell and myeloid cell development by *in vivo* imaging (15, 16). RNA *in situ* hybridization (RISH) is an important tool in molecular cell biology and pathology for the detection and localization of specific mRNA expression within cells and tissues (104). The introduction of non-radioactive probe labeling and detection procedures has greatly facilitated the application of RISH in both research and diagnostics (18). Whole-mount *in situ* hybridization (WISH) has become an important tool for temporal and spatial resolution of gene expression in zebrafish embryos (19). In this study, we use RISH as an alternative way to localize ILLR genes expressed in zebrafish tissues.

Sucessfully constructing stable transgenic zebrafish lines that retain the transgenes has been difficult (20). In order to overcome this problem, transgenesis by *I-SceI* meganuclease was employed in this study. *I-SceI* meganuclease recognizes an 18-bp sequence with little tolerance of degeneracy (21). ILLR transgenes were flanked by two *I-SceI* meganuclease

recognition sites, and co-injected with the *I-SceI* meganuclease enzyme into zebrafish embryos at the one cell stage. The injected embryos were evaluated for GFP expression to select germline transmitting founder zebrafish. A recent study shows that the *Tol2*-mediated transposition system promotes chromosomal integration in zebrafish germline cells (22). The *Tol2* element was first identified from the medaka fish (*Oryzias latipes*), a small freshwater teleost inhabiting East Asia. In this method, a transposon-donor vector and synthetic *Tol2* mRNA were co-injected into one cell stage zebrafish embryos. The translated Tol2 protein catalyzes excision of the transposon construct from the donor vector resulting in the integration of the excised DNA into the zebrfish genome. The germline transmission frequency by the *Tol2* transposon system is usually higher than those mediated by *I-SceI* meganuclease enzyme (23). In order to track ILLR cellular expression patterns, both of these methods were utilized to build stable transgenic zebrafish lines. Understanding the expression pattern of CLRs may promote an understanding of their functional roles in fish immunity, as well as in evolutionary pathways for C-type lectin receptors.

Materials and Methods

Animals. Adult AB Zebrafish were maintained in recirculation water at 28 ^{0}C, on a 10-h light / 14-h dark cycle and fed twice daily. For dissection experiments, zebrafish were euthanized in 0.032% tricaine (Sigma). For observing transgenic zebrafish embryos under the fluorescent microscope, zebrafish were anesthetized with 0.016% tricaine (Sigma). Zebrafish

were handled according to the guidelines from the Institutional Animal Care and Use Committee.

Synthesis of RNA probes. Segments of *illr1*, *illr3*, and *illrL* cDNA sequences (300 - 500 bp) were amplified with gene specific primers (Table 1) and PCR products were cloned into the pGEMT-easy vector (Promega) and sequenced to verify the insert orientation within the vector. For sense probes, plasmids were linearized with *SalI* and, for antisense probes, with *NcoI*. A *TCRα* sequence was used as a positive control (cloned into pBluesriptSK from Dr. Nikolaus Trede): this plasmid was cut with *EcoRI* for a sense probe and *HindIII* for an antisense probe. Digoxigenin-labeled RNA was transcribed *in vitro* using a Dig RNA labeling kit according to the manufacturer's instructions (Roche) with corresponding RNA polymerases to generate either sense or anti-sense probes (Table 2).

RNA *in situ* hybridization (RISH). RISH was carried out on both frozen and paraffin-embedded tissue sections as described before (24, 25). RISH has been performed with modifications of protocols obtained from four different labs. Each protocol differs from the others in several steps. Generally speaking, frozen sections were directly put into 4% paraformaldehyde (PFA, Electron Microscopy Sciences, Hatfield, PA) at 4 ^{0}C for 10-15 min before permeabilization with 10 µg/ml proteinase K (Fisher Scientific) or 0.3% Triton X-100 detergent (Fisher Scientific). Parafin-embedded slides were dewaxed in xylene and then washed with ethanol, diethylpyrocarbonate (DEPC, Sigma-Aldrich, St. Louis, MO)-treated water or PBS. Slides were incubated with hybridization buffer alone (no RNA probe) at 70

^{0}C for 2 hours and then transferred to hybridization buffer containing sense/antisense probes. Slides were washed, blocked, and incubated with an alkaline phosphatase (AP) conjugated anti-DIG antibody (Roche) overnight at 4 ^{0}C. NBT/BCIP substrates were added to slides for color development. Coverslips were mounted using Immunomount (ThermoShandon; Pittsburgh, PA). RISH with the *TCR α-chain* probe was performed in Dr. Gary Litman's lab (University of South Florida).

Cloning ILLR promoters and construction of pILLR:EGFP plasmids. Different lengths of the ILLR putative promoter regions (2 kb, 5 kb, and 10 kb) 5' of the translational starting site were amplified with corresponding 5'/3'overhang primers (Table 3) from bacterial artificial chromosome (BAC) CH211-133n4 and CH211-173p18 using either Elongase Enzyme Mix kit (Invitrogen) or HotMaster Taq DNA Polymerase (Eppendorf). PCR products were digested with *SalI* / *SfiI* and cloned into a pBluescript vector containing the enhanced GFP gene (pSCE:EGFP) and two flanking *I-SceI* recognition sites to build the following plasmids: *illr1^{2k}:EGFP, illr1^{5k}:EGFP illr1^{10k}:EGFP, illr3^{2k}:EGFP, illr3^{5k}:EGFP, illrL^{2k}:EGFP, illrL^{5k}:EGFP, illrL^{10k}:EGFP* (with the superscript indicating the length of the putative promoter). The promoter sequences were confirmed by restriction enzyme digestion (*SalI / SfiI*) and partial DNA sequencing. Plasmid DNA was purified using a Promega miniprep kit (Promega). Primers used in this experiment are listed in Table 3.

Cloning ILLR promoter and construction of pILLR:mCherry plasmids. The putative promoters (~2 kb) of *illr3* and *illrL* were amplified by PCR using Elongase Enzyme Mix kit

(Invitrogen) and the BACs mentioned above. The forward and reverse primers contained attB4/attB1 sequences (Table 3). The final transgene vectors for micro-injection (*illr3*^{2k}*:mCherry* and *illrL*^{2k}*:mCherry*) were built using the MultiSite Gateway® Pro 3.0 kit (Invitrogen). The first step to Gateway® cloning was to insert the 2 kb ILLR putative promoters into the entry vector pDONRP4-P1r (Invitrogen) through a "BP" recombination reaction. The second step was to generate the final transgene vectors through a "LR" recombination reaction which includes four different plasmids: 1) a 5' ILLR promoter entry vector, 2) a middle entry vector encoding the membrane bound red fluorescent protein mCherry(PME-mCherryCAAX), 3) a 3' entry vector encoding a polyA signal sequence (p3E-polyA), and a destination vector that includes a GFP expression cassette (driven by the cardiac myosin light chain promoter) and flanked by Tol2 recognition sites (pDestTol2CG2). The PME-mCherryCAAX, p3E-polyA and pDestTol2CG2 vectors were a generous gift from Dr. Chi-Bin Chien (University of Utah). Primers used in this experiment are listed in Table 3.

Microinjection of transgenes. One cell stage zebrafish embryos were prepared in an agar gel plate for injection as described before (26). For *I-SceI* mediated transgenics, the injection solution included 30 ng/µl plasmid DNA, 0.1% phenol red, 0.5 X I-SceI buffer, 0.3 U/ µl *I-SceI* Meganuclease (27). For *Tol2* mediated transgenics, the injection solution included 20 ng/µl DNA, 0.1% phenol red, 25 ng/µl *in vitro* transcribed and 5' capped *Tol2* transposase mRNA. The *Tol2* transposase mRNA was generated using the mMESSAGE mMACHINE SP6 Kit (Ambion, Inc) from the PCS2FA-transposase vector (gift from Dr. Chi-Bin Chien, University of Utah) (28).

Fluorescence microscopy. Zebrafish embryos (3-14 dpf) that were injected with transgenes were evaluated for GFP or mCherry fluorescence using a Leica DM5000B compound microscope. For transgenic (Tg) zebrafish (*illr3*^{2k}*:mCherry* and *illrL*^{2k}*:mCherry*) screening, only those founders with GFP-positive hearts were selected for future study.

Genotyping ILLR:EGFP transgenic fish. Genomic DNA from either founder larvae (F_1) or F_1 adult tail fin was prepared according to previous methods (29). Individual ILLR:EGFP F_0 fish were out-crossed to wild type zebrafish to identify F_0 positive zebrafish that incorporated the transgene into their germline. Founder larvae (F_1) from a single mating were grown to 5 days old and euthanized for genotyping. Transgene positive F_0 zebrafish were out crossed to wild type zebrafish to generate F_1 transgenic generation. Once the F_1 generation grew to sexual maturity, zebrafish were anesthetized with 0.016% tricaine (Sigma) and a small piece of the tail fin was excised with sterilized scissors. Fin samples were placed into a 1.5 ml Eppendorf tube containing 100 μl of 50 mM NaOH. The samples were heated to 95 ^{0}C for 15-20 min until the tissue was completely dissociated. After the sample was cooled to 4 ^{0}C on ice, 1/10th volume of 1 M Tris-HCl (pH 8.0) was added to neutralize the basic solution. Samples were centrifuged at 13,000 rpm for 1 min to pellet debris and permit the recovery of the supernatant for PCR genotyping. Genomic PCR was performed using forward primers complementary to the ILLR promoter region and a reverse primer complementary to the GFP transgene sequence, which would generate a 900 bp product (Table 3). F_1 positive zebrafish were crossed with transgenic siblings to generate F_2 offspring.

Flow cytometry. Cell collection and flow cytometry were performed on spleen and kidney samples from *illr1*^{10k}*:EGFP* transgenic zebrafish as described previously (30). In brief, zebrafish were euthanized and spleen and kidneys were dissected and dissociated by teasing through a 40 μm cell strainer (BD Falcon) into ice-cold 0.9 x PBS plus 5% fetal bovine serum (FBS). Cells were fixed in 4% paraformaldehyde (Fisher) at 4 ^{0}C for 15 min before permeabilizing in 10 μg/ml proteinase K (Fisher) at 37 ^{0}C for 30 min. Cells were incubated with an anti-GFP mouse monoclonal antibody (Roche) at 4 ^{0}C for 30 min first and then incubated with an allophycocyanin (APC) conjugated goat anti-mouse IgG secondary antibody (Roche) at 4 ^{0}C for 30 min. Labeled cells were washed and subjected to flow cytometric analysis (BD FACSCalibur™: BD Biosciences; San Jose, CA).

Reverse transcriptase PCR (RT-PCR). Kidney and spleen tissues from wild type and F_1 *illr1*^{10k}*:EGFP* transgenic zebrafish were homogenized with a pellet pestle motor (Kontes Fisher). Total RNA (2 μg) was reverse transcribed with oligo dT in 20 μl reactions and diluted to 100 μl with dH_20. GFP primers (Table 3) were designed to amplify its partial cDNA. PCRs were incubated at 94 ^{0}C for 2 min, followed by 35 cycles of 94 ^{0}C for 30 s, 55 ^{0}C for 30 s, and 72 ^{0}C for 30 s. A positive control PCR utilized the *illr1*^{10k}*:EGFP* plasmid as template. β-actin was amplified with 25 cycles at the annealing temperature 65 ^{0}C. RNA extraction and cDNA synthesis are described in detail in Chapter 3.

Results

ILLR expression in tissue section

In order to localize and detect ILLR specific expression in morphologically preserved tissue sections, four different RISH protocols were attempted. Initially, RISH using *illr1*- and *illr3*-specific probes on paraffin-embedded sections was performed using a Biochain Institute kit (Hayward, CA) (Fig. 1). Multiple repeats on paraffin-embedded tissue sections from spleen, kidney, and intestine have been done in order to detect cells expressing *illr1* and *illr3*. Either no signal or a false positive (purple) signal was detected for *illr1* (Fig. 1A). *Illr3* positive cells observed in occasional spleen samples (Fig. 1B) were not reproduced with multiple repeats. This led us to utilize frozen tissue sections which can provide a higher quality and quantity of RNA signal than paraffin-embedded sections (31). Cryostat sections of PFA-fixed tissues which have been immersed in sucrose provide excellent visualization of mRNA. Unfortunately, the *illrL* anti-sense probe appears to non-specifically bind RNA in kidney tissue which had been previously shown by RT-PCR to express higher levels of *illrL* compared to *illr1* and *illr3* (Fig 2) (11). The same phenomenon was seen for *illr3* when a GeneDetect (Bradenton, FL) oligonucleotide probe was used on frozen sections (Fig. 3). The TCR α probe was included in this study as a positive control probe to verify that the protocol we followed with the ILLR probes works for other probes (e.g. as a positive control) (32). TCR α positive cells were identified in the antisense reaction, but not with the sense probe

(Fig. 4). However, further confirmation of their identity as T cells is still needed. Overall, we did not successfully identify any ILLR positive cells with these techniques.

Generation of stable *illr1*^{10k}*:EGFP* transgenic zebrafish

In order to construct *illr1* transgenic zebrafish line, different lengths (2 kb, 5 kb, and 10 kb) of the putative *illr1* promoter region were amplified and cloned into pSCE-EGFP vector (Fig. 5). The flow diagram in Figure 6 shows the plasmid structure for *illr1*^{10k}*:EGFP* as an example of and procedures for building the transgenic line (*illr1*^{10k}*:EGFP*). Briefly, 1-cell embryos were injected with the *illr1*^{10k}*:EGFP* transgene. Embryos at 3-14 dpf were observed under the fluorescent microscope to identify detectable GFP expression in circulating blood cells as described (33). Unfortunately, no visible GFP was seen during that period of embryonic development. The potential founder (F_0) larvae were grown to the adult stage and 16 individual adult zebrafish were out crossed to wild type fish. F_1 embryos from these crosses were subjected to genomic PCR genotyping. This strategy allows us to determine which F_0 zebrafish are transmitting the transgene to the progeny. One male and one female transgene-positive F_0 zebrafish were identified through this strategy (Fig. 7A). These F_0 individuals were crossed again with wild type zebrafish and the resulting F1 embryos were raised to maturity. Fin-clip genotyping from the F_1 individuals (Fig. 7B) identified 29 out of 110 offspring to be transgene positive (Table 4). These positive F_1 zebrafish were mated with siblings to obtain the F_2 generation before they were euthanized for Flow cytometry and RT-PCR analysis. Overall, 2 positive founder (F_0) and 29 F_1 zebrafish were identified carrying

the transgene *illr1*^{10k}*:EGFP*. One positive founder (F_0) zebrafish for the *ill3*^{2k}*:EGFP* transgenic line was identified as well (Table 4).

Generation of *illr3*^{2k}*:mCherry* and *illrL*^{2k}*:mCherry* transgenic zebrafish

Based on the observation that GFP was not detectable in *illr1*^{10k}*:EGFP* transgenic zebrafish embryos under the fluorescent microscope, the *Tol2* transposon-based trasngenesis system was used to develop *illr3* and *illrL* transgenic lines. The major advantages of this Tol2-based transgenesis strategy are: 1) a GFP^+ heart provides an extremely convenient way to screen transgenic zebrafish compared with the genomic PCR genotyping and 2) on average, the *Tol2* transposon system has a higher efficiency of germline transmission over *I-SceI* meganuclease-mediated tansgenesis (23, 34). Previous studies have shown that about 50% of zebrafish co-injected with a plasmid-based transgene flanked by Tol2 recognition sites and *Tol2* transposase mRNA incorporated the transgene into their genome and transmitted the transgene to their progeny (22). With this method, transgenic lines for *illr3*^{2k}*:mCherry* and *illrL*^{2k}*:mCherry* were generated. F_1 embryos at 3-14 dpf were observed under the fluorescent microscope to identify detectable mCherry expression in circulating blood cells as described (33). There was no visible mCherry seen in embryos obtained during that period after fertilization. However, the larvae from both F_0 transgenic zebrafish lines at 5 dpf showed their heart beating with GFP (Fig. 8). Data on flow cytometric analysis are described in Chapter 4. Overall, the *Tol2* transposon system provides a convenient tool to build transgenic zebrafish lines for *illr3* and *illrL*.

Flow cytometry and RT-PCR analysis on *illr1^{10k}:EGFP* F_1 transgenic zebrafish

To test if GFP positive cells can be identified from *illr1^{10k}:EGFP* transgenic zebrafish via Flow cytometry, 29 F_1 zebrafish were euthanized. Cells were confined into lymphoid (R1) and myeloid (R2) population based on forward and side scatter scales (30). GFP-positive cells from the transgenic zebrafish kidney consisted of 4.88% of R1 population (Fig. 9B) compared to 5.2% from wild type zebrafish kidney (Fig. 9A). Antibody staining against GFP showed a very low percentage of GFP positive cells (0.28% in transgenic zebrafish and 0.42% in wild type zebrafish), suggesting that auto-fluorescence in zebrafish kidney cells interferes with the extremely low level of cells bound by anti-GFP Abs. A similar result was seen in the R2 population where GFP positive cells from the transgenic zebrafish and wild type kidney were 4.2% and 3.19% respectively. In the transgenic zebrafish kidney cells, 0.1% of the R2 gated cells were APC-positive while wild type zebrafish kidney had 0.18% positive cells. However, RT-PCR analysis of cDNA samples from kidney and spleen tissues did not detect GFP transcripts (data not shown). These observations suggest that GFP is not transcribed in the transgenic zebrafish and that the observed APC-positive cells result from background staining. These results indicate that we are unable to isolate GFP+ cells from the *illr1:EGFP* transgenic zebrafish using flow cytometry.

Discussion

The results shown here unexpectedly failed to identify ILLR expressing cells by RISH. This technique has been reported to be sensitive enough to detect as few as 10 transcripts in a single cell (35). WISH has been widely applied to localize important genes involved in zebrafish development, whereas fewer reports employ RISH on adult zebrafish tissues. Danilova et al. demonstrated the distribution of mature B and T lymphocytes in adult peripheral organs through RISH (32, 36). Unfortunately, we failed to detect ILLR gene expression with this technique. There were two results from multiple attempts to use RISH to detect ILLR+ cells: no signal or non-specific signal. Based on the data from the positive control probes, TCRα and Pax5, we learned that the protocol from Dr. Litman's lab is the best one among the four protocols evaluated. It is recommended for RISH with other genes. However, ILLR genes are expressed at extremely low levels, making it difficult to directly detect them. Other possible explanations for why the ILLR genes were not detected by RISH include inappropriate length or location of the RNA probe, incorrect hybridization temperature, washing conditions, or substrate incubation time. However, each of these critical conditions has been altered in order to identify optimal conditions for detecting ILLR gene expression without success. An alternative way to identify ILLR expressing cells would be to develop an antibody against each ILLR that would allow us to identify cells expressing ILLR genes using Western blot of flow cytometry techniques.

The zebrafish is emerging as an important vertebrate model of development for many reasons, but functional studies of the immune system have been limited by the lack of reagents to isolate and characterize of different lymphoid or myeloid cell populations. For example, antibodies against cell lineage markers CD4 and CD8 are not yet available. Because of these limitations, studies in zebrafish heavily rely on transgenic technology and on use of gene promoters to drive the expression of GFP (or other fluorescent proteins) in specific cell populations (37-39). Here, the putative promoters for the ILLR genes were cloned into an EGFP transgene vector and transgenic zebrafish were developed. Unfortunately, analysis by flow cytometry of cells from *illr1*^{10k}*:EGFP* transgenic zebrafish indicated that the percentage of GFP positive kidney cells from transgenic zebrafish was comparable to that from wild type zebrafish. Labeling with an anti-GFP antibody showed less than 1% positive cells in transgenic zebrafish kidney tissue, which suggests an extremely low level of GFP expression driven by ILLR promoter. Failure to detect GFP could also be due to use of an incomplete promoter region. Since we did not identify the minimal promoter fragment that is required for *illr1*-specific expression, this 10 kb region above the translation start site may be not enough to recapitulate *illr1* expression. For example, enhancers may be present after the translational start site and may be required to direct normal *illr1* transcription. Recent studies on Ly49 promoters in mice show that there are three promoters (Pro1, Pro2, and Pro3) which control the Ly49 gene family (40). The Ly49 Pro1 element contains bidirectional promoter activity and is identified in all of the inhibitory Ly49 family members (41). Pro3 is a downstream promoter before the first Ly49 coding exon (42). These observations demonstrate that a complicated mechanism may be utilized to regulate NK cell receptor gene

transcription. Further study is necessary to identify an effective promoter region for ILLR gene transcription.

In order to avoid the green fluorescence background in wild type fish, we also built *ILLR:mCherry* transgenic zebrafish which used an mCherry fluorescent marker to help identify ILLR positive cells. In the first two weeks after fertilization we did not observe mCherry positive cells in the circulating blood of these transgenic zebrafish (*illr3*^{2k}*:mCherry* and *illrL*^{2k}*:mCherry*). Therefore, these transgenic zebrafish were raised to adult stage and received immune challenges to boost ILLR gene expression as described in Chapter IV.

Table 1. List of primers to synthesize ILLR RNA probes.

Primer name	Primer sequence
Illr1_probe_F	TGTGCTGTCCGCTGGACTCATTCT
Illr1_probe_R	ACAGTAAGCATCTGTCCAGAAATC
Illr3_probe_F	CTTGGAGGTCTCTGTGCTGTA
Illr3_probe_R	TTGGCTCAGTGGCTGGTTGTC
illrL_probe_F	TCAGCAGTGCTCGAACACATA
illrL_probe_R	TGCTTATCTGGAGGATCTGGT

Table 2. List of RNA *in situ* hybridization probes.

Probe name	Size (bp)	Plasmid (Amp^R)*	Sense/Promoter	Antisense/Promoter
Illr1 (JY0216)	375	pGEMT-EASY	*Sal*I/T7	*Nco*I/SP6
Illr3 (JY0217)	375	pGEMT-EASY	*Sal*I/T7	*Nco*I/SP6
IllrL (JY0132)	500	pGEMT-EASY	*Sal*I/T7	*Nco*I/SP6
TCRα	450	pBlueSK	*Eco*RI/T7	*Hind*III/T3
Pax5 (B cell)	1000	pBlueSK	*Hind*III/T3	*Not*I/T7

* All the plasmids are AMP resistant.

Table 3. List of primers used in generation of transgenic zebrafish lines.

Purpose	Primer name	Primer sequence
illr1:EGFP	Illr1_SfiI_2k_F	GACTGGCCAAGTCGGCCTCAGAATGATAGCACCAGTAGTTTC*
	Illr1_SfiI_5k_F	GACTGGCCAAGTCGGCCACATACGCAATGGTGAGTCTGGAT
	Illr1_SfiI_10k_F	GACTGGCCAAGTCGGCCAGTTACTAATTGCAGTGTTGCA
	Illr1_SalI_R	GATCGTCGACCAGATTAACAGTCTTACTTCTGAGTCTCCT
illr3:EGFP	Illr3_SfiI_2k_F	GTCAGGCCAAGTCGGCCATGTTTGAGTGTTAGAAGACCA
	Illr3_SfiI_5k_F	GTCAGGCCAAGTCGGCCAAACATGCAAACTCCACACAGA
	Illr3_XhoI_R	GATCCTCGAGGAACTTGAGACTTTGCTGAGATTTGAGCGC
illrL:EGFP	IllrL_SfiI_2k_F	GTCAGGCCAAGTCGGCCAAACCTCAGTGAACTCTTCTGC
	IllrL_SfiI_5k_F	GTCAGGCCAAGTCGGCCTCACCTGAATTTACCACAGGTC
	IllrL_SfiI_10k_F	GACTGGCCAAGTCGGCCAGTTACTATTGCAGTGTGTTGCA
	IllrL_SalI_R	GATCGTCGACGCTTCTGGAGGGATGCTGCTCAAAGCCTGT
illr3^{2k}:mCherry	Illr3_2k_ttB4_F	GGGGACAACTTTGTATAGAAAAGTTGATGTTTGTTAGAAGACCA
	Illr3_attB1_R	GGGGACTGCTTTTTTGTACAAACTTGGAACTTGAGACTTTGCTGAGAT
illrL^{2k}:mCherry	IllrL_attB4_F	GGGGACAACTTTGTATAGAAAAGTTGAACCTCAGTGAACTCTTCTGC
	IllrL_attB1_R	GGGGACTGCTTTTTTGTACAAACTTGGCTTCTGGAGGGATGCTGCTCA
Genomic PCR	Illr1_F	AAGTGTGAAACCCCACTCCTC
	Illr3_F	TCCTGACGCTGGCTAATAAGG
	IllrL_F	GTCTAACTTAGATCGTGTGTGC
	GFP_R	GTGGTGCAGATGAACTTCAGG
RT_PCR GFP	GFP_RT_F	ACAGCTCGTCCATGCCGAG
	GFP_RT_R	TGCCCATCCTGGTCGAGCT

*Restriction sites or attB4/attB1 were underlined in individual primer sequence.

Table 4. List of constructed ILLR transgenic zebrafish lines

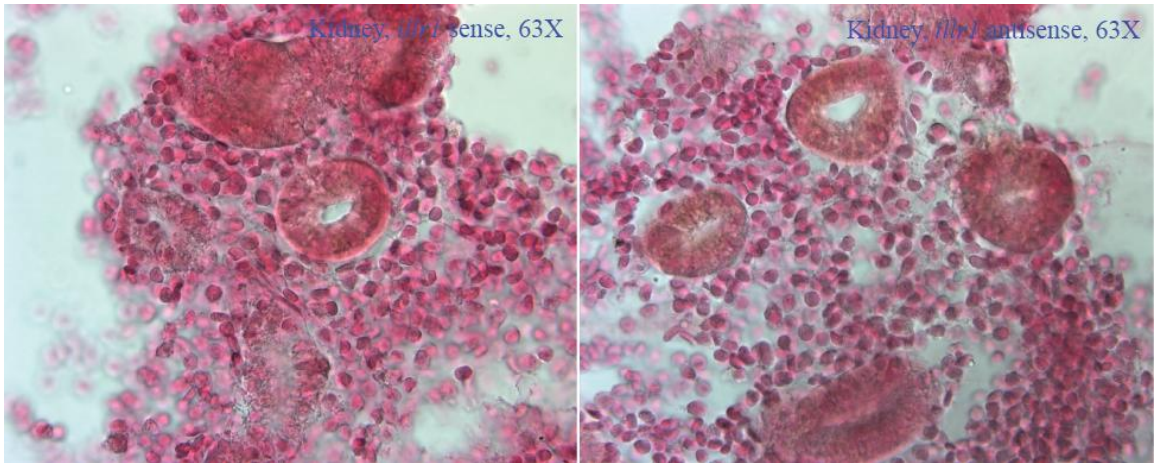
Injected Plasmid	Injection/mating date	Survival adult fish#	Genotyping (Number of positive fish)	Notes
illr1_10k:EGFP	10/12/06	16	2	
illr1_5k:EGFP	1/15/07	11	0	Ctrl 15
illr3_2k:EGFP	12/29/06	20	1	Ctrl (12/2/06) 14
illr3_5k:EGFP	3/31/07	9	0	
illrL_2k:EGFP	1/4/07	15	0	Ctrl 13
illrL_5k:EGFP	1/3/07	11	0	
illrL_10k:EGFP	6/21/07	15	0	Ctrl 8
illr1_10k:EGFP F1 Male	4/10/07 6/4/07 6/17/07 7/17/07	20 10 15 15	10	
illr1_10k:EGFP F1 Female	3/10/07 6/4/07 6/17/07	5 20 10	19	F2 generation is growing
illr3_2k:EGFP F1	7/17/07	25	1	
illrL_2k:mCherry	11/10/07 11/21/07	8 9	Performed flow, 7 left	Total 17 Ctrl (11/21/07) 15
illr3_2k:mCherry	11/7/07 1/17/08	2 40	Mate with wild type	Ctrl (11/16/07) 8 Ctrl (1/07/08) 50
illr3_2k:mCherry F1	2/12/08 3/5/08	7 30	37	

Table 4 Continued

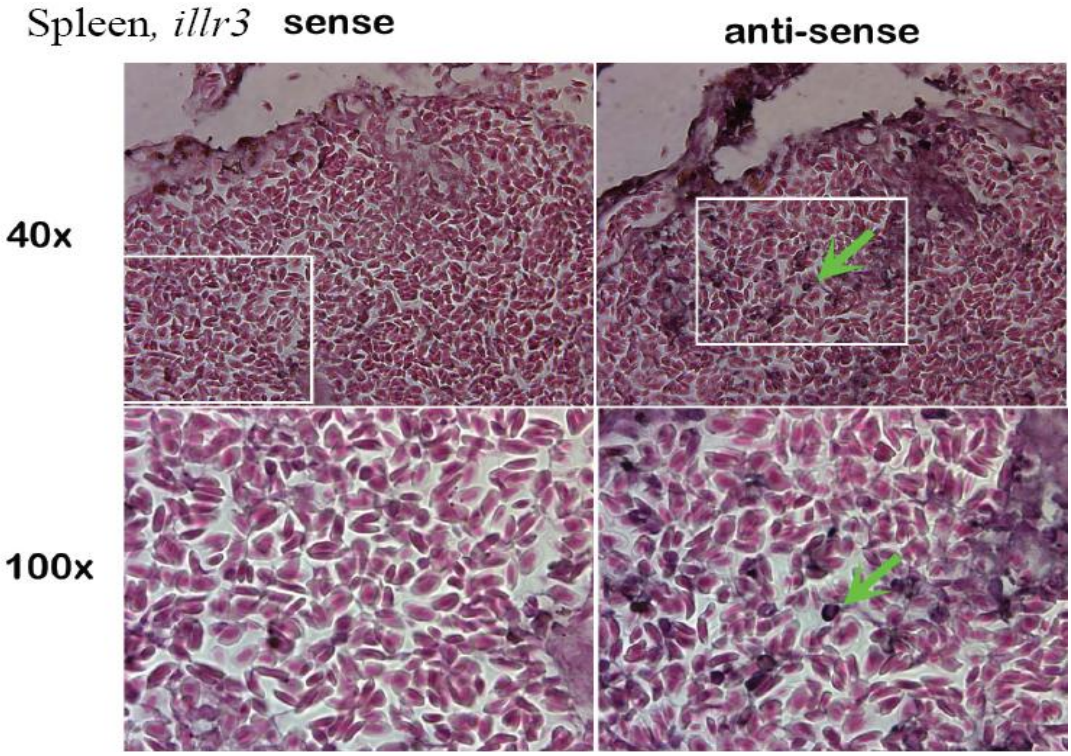
illr1_10k:EGFP F2	2/5/08 2/20/08 2/28/08 3/5/08	12 12 16 25		Ctrl (2/20/08) 15

Figure 1. Zebrafish *illr1* and *illr3* expression. RISH was performed with Biochain Institute kit on paraffin-embedded tissue sections to detect (A) *illr1* on kidney and (B) *illr3* on spleen. A digoxigenin (DIG)-labeled antisense RNA probe (right panels) was hybridized to tissue sections and detected via an alkaline phophatase (AP)-conjugated anti-DIG antibody. NBT/BCIP was used as the substrate for AP resulting in a purple color reaction if cells express *illr1* or *illr3*. Cells were counter-stained with nuclear Fast Red. The sense probe is used as a negative control since it cannot hybridize with the target gene mRNA. The 100x images (B) are magnifications of the white rectangles in the 40x images. Green arrows point to the same purple cell (at 40x and 100x) possibly indicating illr3 expression.

A.


Kidney, illr3 sense, 63X
Kidney, illr3 antisense, 63X

B.


Spleen, illr3 sense
anti-sense
40x
100x

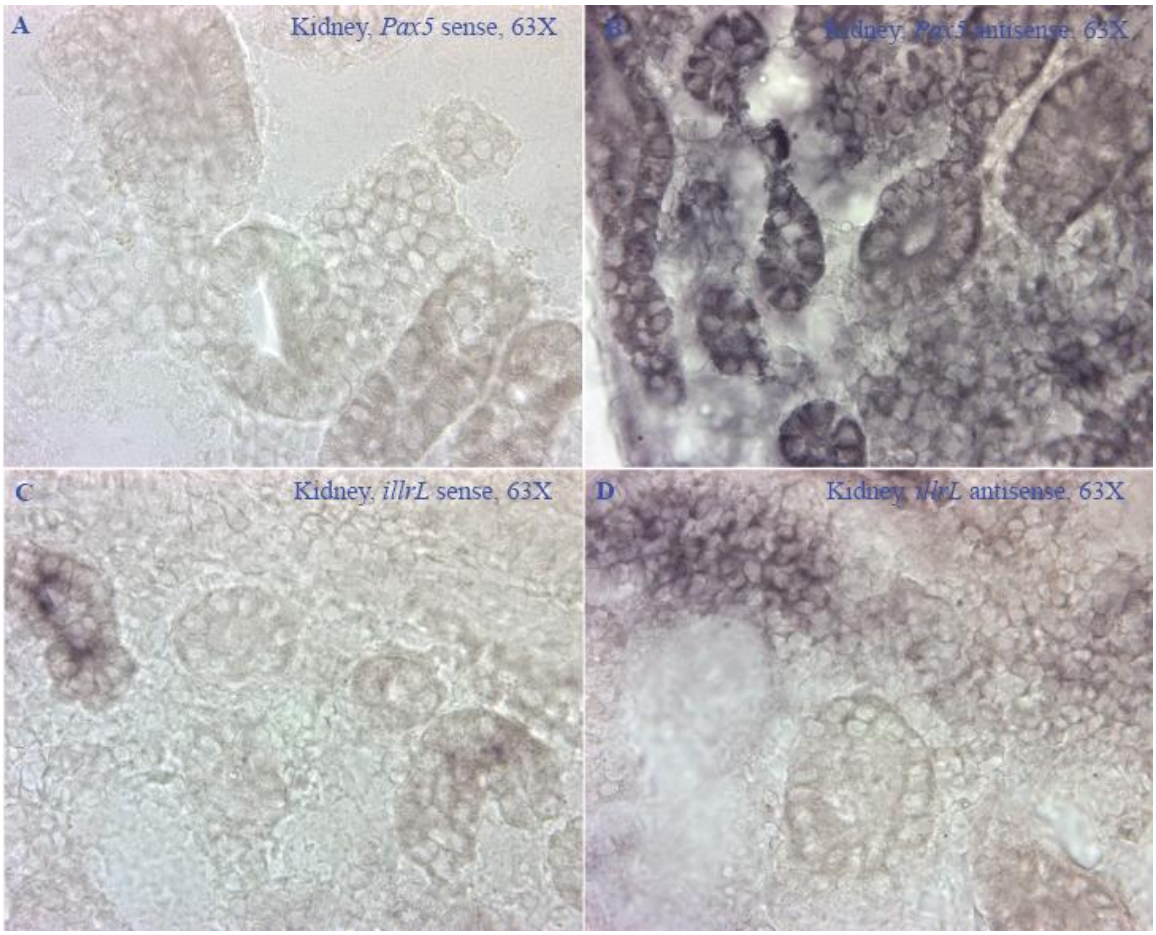


Figure 2. Zebrafish *illrL* expression in kidney tissue section. RISH using a protocol from Dr Michael Schorpp's lab (Max-Planck Institute of Immunobiology, Freiburg, Germany) was performed to detect *illrL* expression on frozen tissue sections in adult zebrafish (43). The upper micrographs (A and B) show the results from a positive control probe *pax5* as a marker of B cell lineage. The lower micrographs (C and D) showed a non-specific reaction for *illrL* expression on kidney.

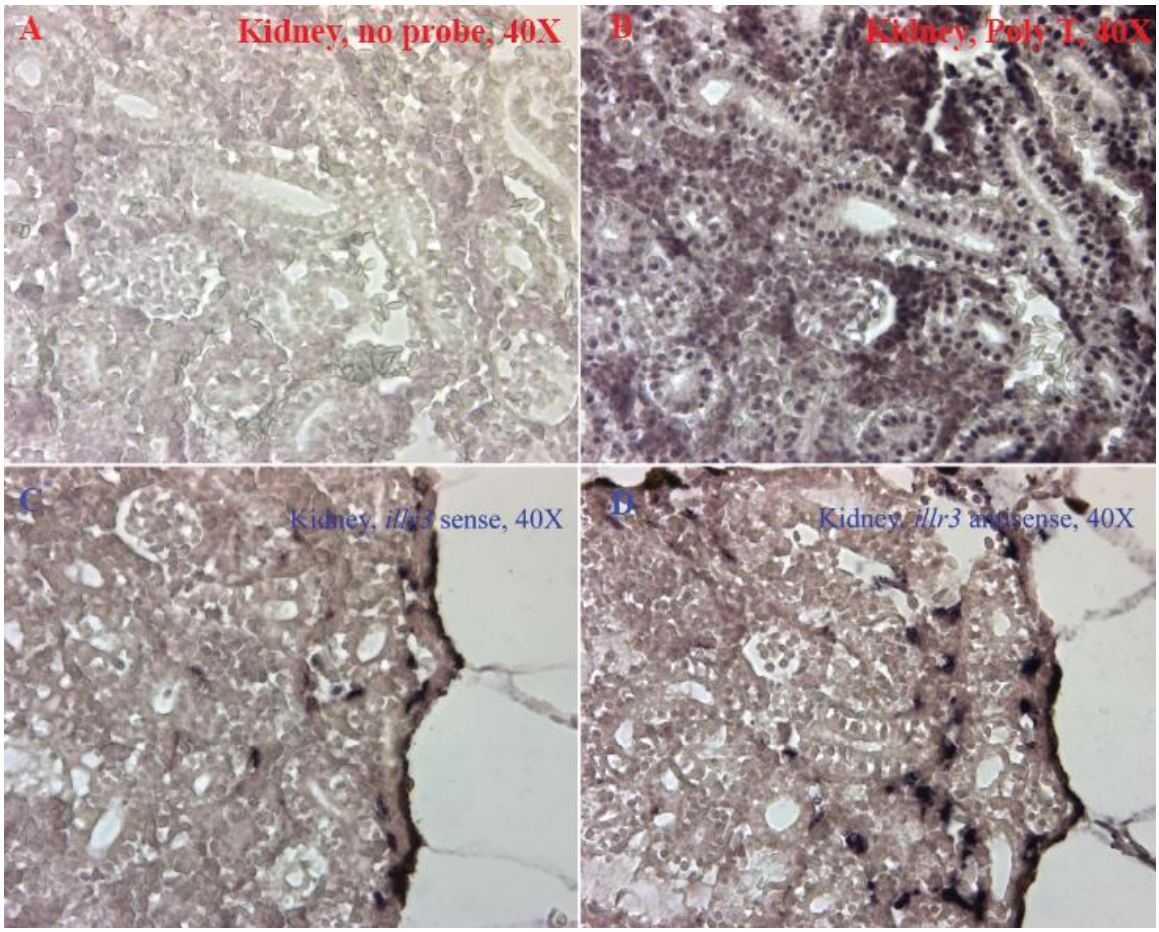


Figure 3. Zebrafish *illr3* expression in kidney. RNA ISH using GeneDetect oligonucleotide probes was carried out on frozen sections. (A) No probe is included as a negative control. (B) A polyT probe was employed to show the RNA quality on frozen tissue sections. (C) Sense and (D) antisense probes were added to demonstrate *illr3* expression on adult zebrafish kidney tissue. Samples were washed directly following NBT/BCIP incubation without counter-staining.

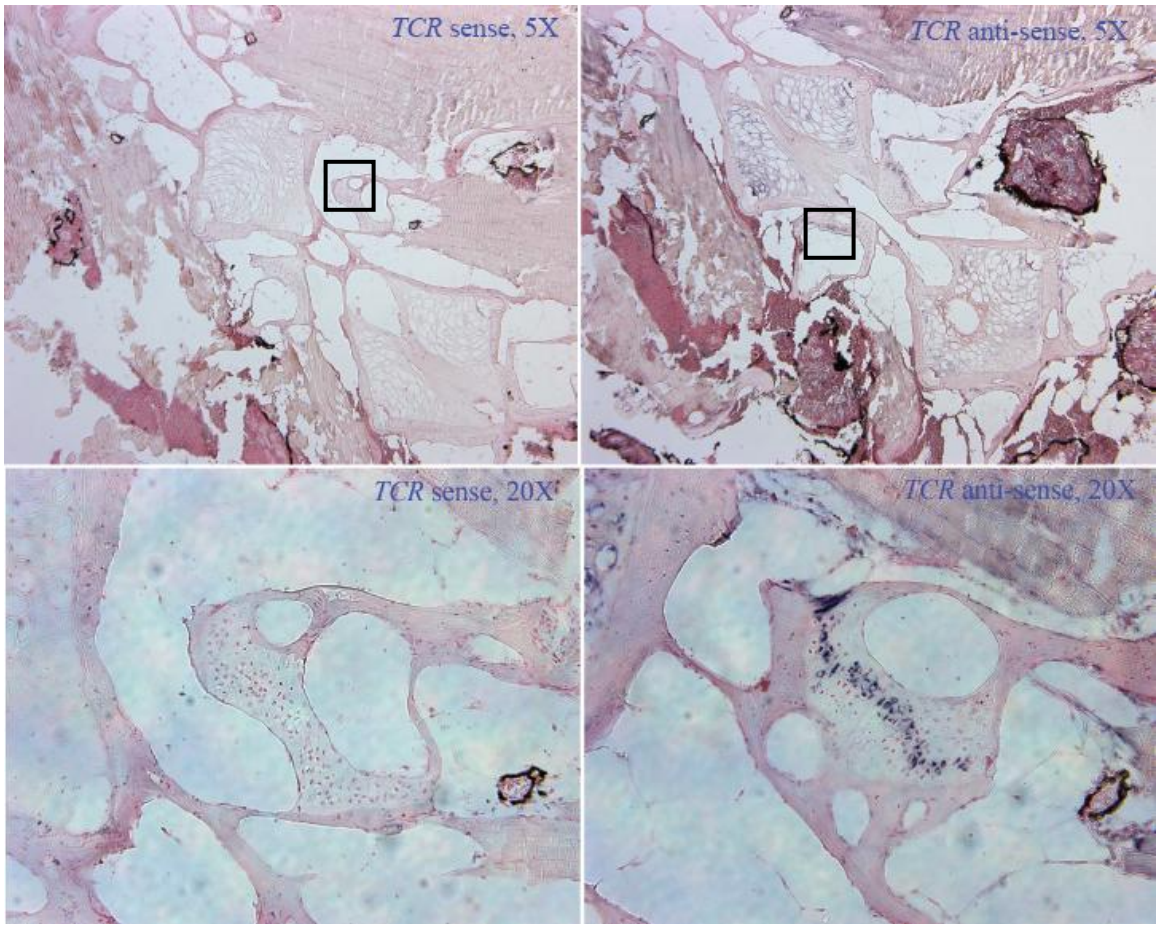


Figure 4. RISH of TCRα on one month old zebrafish tissue section. RISH was performed on paraffin-embedded tissue sections. As a positive control, TCRα sense and antisense probes were used to detect T cells. The lower micrographs are the magnification (20 x) of box areas in the upper micrographs. TCR α positive cells were identified in the antisense reaction, but not with the sense probe.

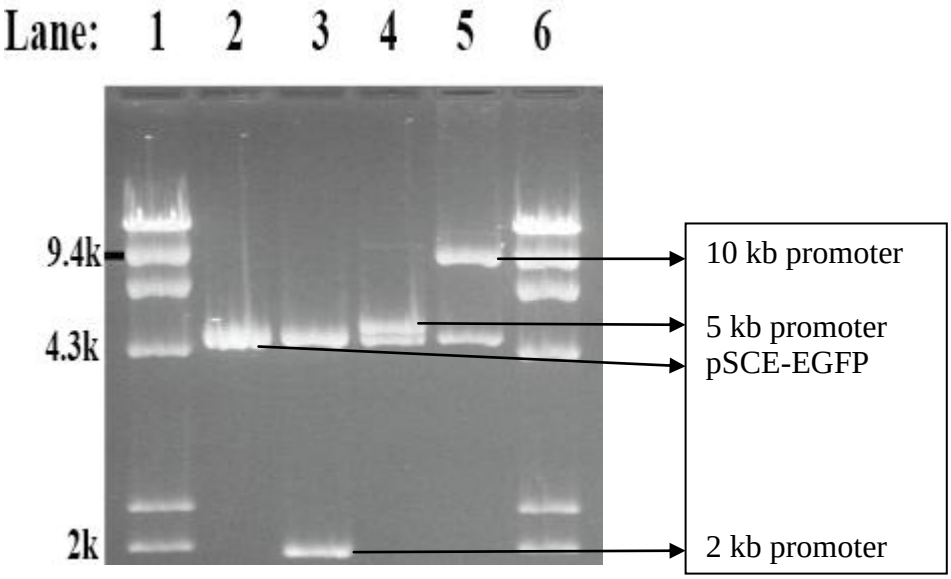


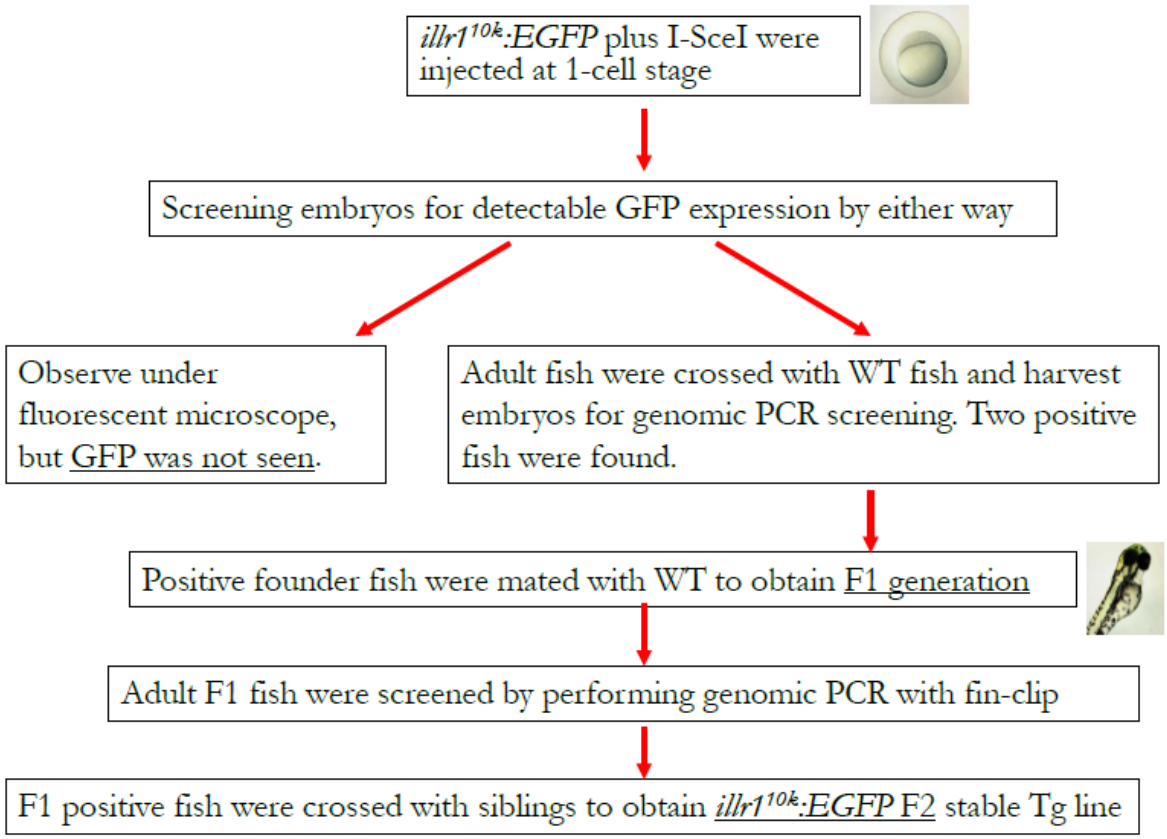
Figure 5. Restriction enzyme analysis of *illr1:EGFP* plasmids. Restriction enzymes *SfiI* and *SalI* were used to digest plasmids pSCE-EGFP (Lane 2), *illr1*^{2k}*:EGFP* (lane 3), *illr1*^{5k}*:EGFP* (lane 4), *illr1*^{10k}*:EGFP* (lane 5). Lame 1 and 6 are λ *HindIII* DNA marker.

Figure 6. Strategy to build *illr1*^{10k}*:EGFP* transgenic zebrafish line. (A) Diagram of the injected plasmid *illr1*^{10k}*:EGFP* is shown. "P" means promoter. (B) This flowchart outlines the procedures to make F_2 stable transgenic lines for ILLR genes. The transgene (for example, *illr1*^{10k}*:EGFP* plasmid as shown in A) was co-injected with *I-SceI* meganuclease into the cytoplasm of 1-cell stage embryos. Embryos at 3-14 dpf were observed under the fluorescent microscope to identify detectable GFP expression in circulating blood cells. Otherwise, zebrafish were grown to adult and mated with wild type zebrafish. Genomic DNA from their embryos were isolated and analyzed by genomic PCR to detect transgenes. These F_0 positive individuals were crossed again with wild type zebrafish and the resulting embryos were raised to maturity as F_1 generation. F_1 individuals were screened by Fin-clip genotyping. The positive F_1 zebrafish were mated with siblings to obtain F_2 stable transgenic lines.

A.


SceI
2kb, 5kb, or 10kb ILLR P
EGFP
SceI

B.


illr1^10k:EGFP plus I-SceI were injected at 1-cell stage
Screening embryos for detectable GFP expression by either way
Observe under fluorescent microscope, but GFP was not seen.
Adult fish were crossed with WT fish and harvest embryos for genomic PCR screening. Two positive fish were found.
Positive founder fish were mated with WT to obtain F1 generation
Adult F1 fish were screened by performing genomic PCR with fin-clip
F1 positive fish were crossed with siblings to obtain illr1^10k:EGFP F2 stable Tg line

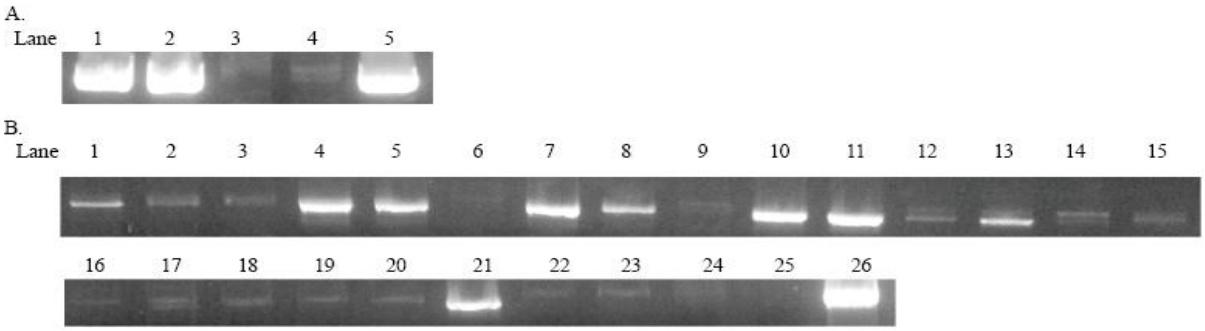


Figure 7. Screening transgenic zebrafish (*illr1*^{10k}*:EGFP*) by genomic PCR. (A) The founding F_0 generation zebrafish were mated with wild type zebrafish individually and genomic DNA from the resulting embryos were subjected to PCR-based genotyping. Lane 1: PCR from embryos derived from a male zebrafish injected with *illr1*^{10k}*:EGFP*, 2: PCR from embryos derived from a female zebrafish injected with *illr1*^{10k}*:EGFP*, 3: PCR from embryos derived from a female zebrafish injected with *illr1*^{10k}*:EGFP* that did not incorporate the transgene into its germline, 4: PCR from wild type embryos, 5: PCR positive control with *illr1*^{10k}*:EGFP* plasmid. The two positive F_0 zebrafish were crossed with wild type zebrafish and the embryos were grown up to adult stage named as F_1 generation. In Fig B, PCR genotyping was conducted using tail fin genomic DNA from adult F_1 zebrafish. Samples from lane 1 to lane 23 represent genotyping of individual F_1 zebrafish. Lane 24: genotyping from wild type zebrafish, 25: negative control without template, 26: positive control PCR with the *illr1*^{10k}*:EGFP* plasmid. Strong bands in Lane 4, 5, 7, 8, 10, 11, and 21 indicated a positive F_1 zebrafish. Twenty-nine F_1 zebrafish were identified as F_1 transgenics from 110 zebrafish in this way.

A.

B.

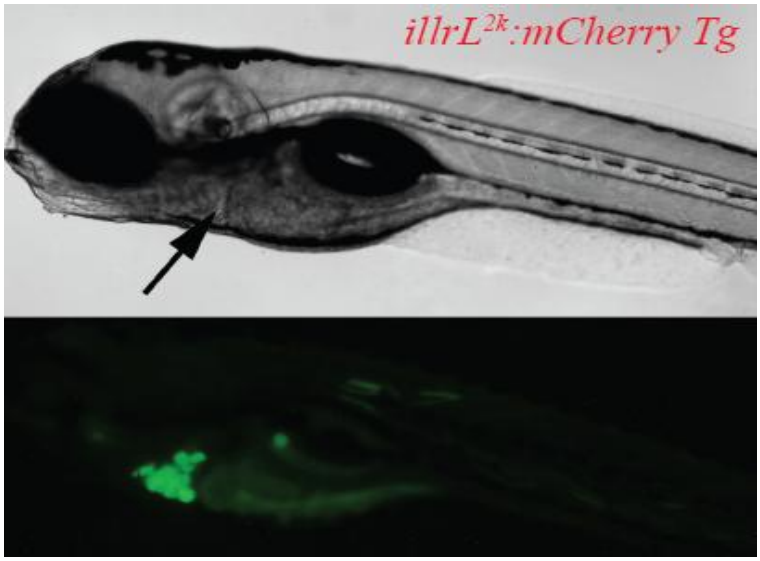


Figure 8. *illrL^{2k}:mCherry* transgenic zebrafish. (A) Diagram of the injected plasmid *illrL^{2k}:mCherry* was shown here. It was built with a MultiSite Gateway Pro 3.0 kit (Invitrogen) (28). Its sequence was confirmed by restriction enzyme digestion analysis and DNA sequencing. "P" means promoter. (B) Founder F_0 zebrafish larvae at 5 dpf were observed under the fluorescent microscope. The arrow points where the green heart locates on the bright field. GFP hearts were also seen in *illr3^{2k}:mCherry* transgenic zebrafish (data not shown here).

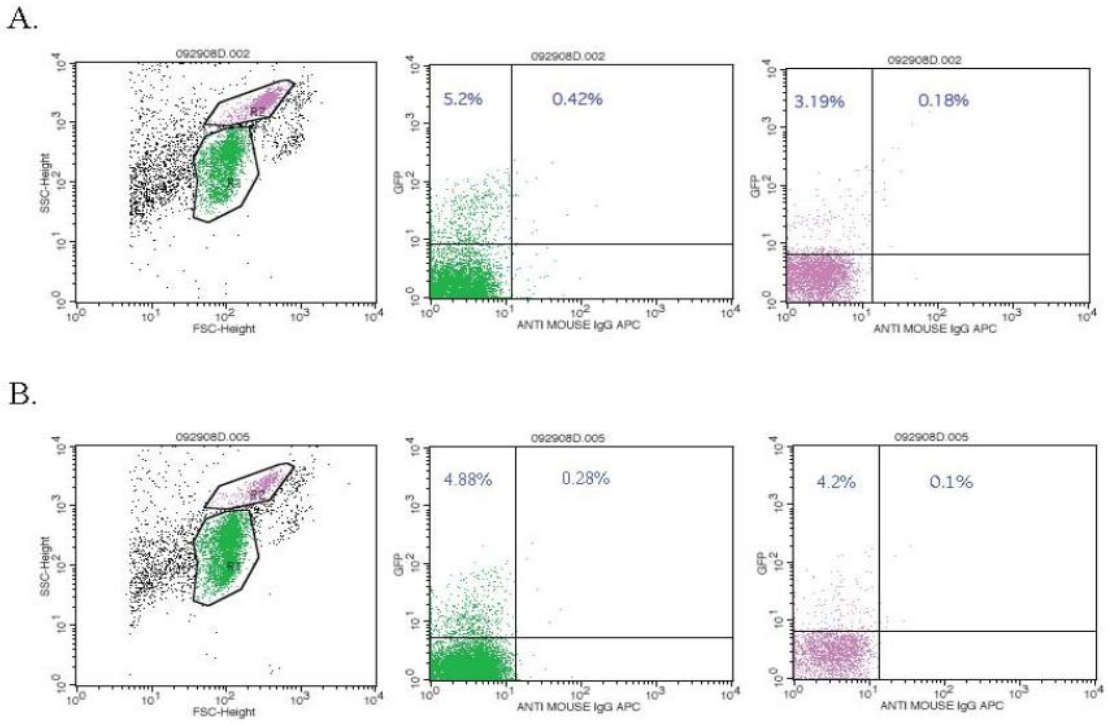


Figure 9. Analysis of F_1 transgenic zebrafish (*illr1*^{10k}*:EGFP*) by FACS. (A) Ten wild type and (B) 10 transgenic zebrafish were euthanized and dissected. Cell suspensions from the kidney were filtered by teasing through a 40 µm cell strainer into ice-cold PBS plus 5% FBS. Cells were fixed in 4% PFA before permeabilization in 10 µg/ml proteinase K. Cells were incubated with an anti-GFP mouse monoclonal Ab, washed, and then incubated with an allophycocyanin (APC) conjugated goat anti-mouse IgG secondary antibody. Labeled cells were washed and then subjected to flow cytometric analysis as described before (30). R1 population (green) represents zebrafish lymphoid cells and R2 population (purple) represents myeloid population.

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CHAPTER 3. Transcriptional response of immune-related, lectin-like receptor (ILLR) genes to immune challenge

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Contribution by our Collaborators:

• SHRV infection study was performed in Dr. Carol Kim's lab. I participated in the *S. iniae* infection study in Dr. Melody Neely's lab.

Transcriptional response of immune-related, lectin-like receptor (ILLR) genes to immune challenge

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Abstract

Immune-related, lectin-like receptor (ILLR) genes encode Group II C-type lectin domain (CTLD) receptors but possess signaling motifs reminiscent of Group V (natural killer cell) CTLD receptors. Three ILLR genes (*illr1*, *illr3* and *illr4/illrL*) and one pseduogene (*illr2*) are encoded on zebrafish chromosome 19. ILLR genes display variable expression within the lymphoid and myeloid lineages: *illr1* transcripts are detected in the lymphoid lineage, *illr3* transcripts are detected in both the lymphoid and myeloid lineage and *illr4* transcripts are detected in the myeloid lineage. The CTLD sequences and overall protein structure of the ILLRs are similar to multiple mammalian innate immune receptors such as macrophage-inducible C-type lectin (Mincle/Clec4e/Clecsf9) and dendritic cell-specific ICAM grabbing non-integrin (DC-SIGN/CD209). As Mincle was originally identified by its transcriptional response to immune stimuli, the goal of this study was to determine if the ILLR genes are transcriptionally responsive to immune challenge. Adult zebrafish were infected with either bacteria (*Streptococcus iniae*) or virus (Snakehead rhabdovirus) and the transcriptional levels of the ILLR genes were monitored in multiple tissues by quantitative RT-PCR. Relative levels of *illr4* transcripts in the spleen were dramatically increased in response to both bacterial and viral challenge. In contrast, no dramatic transcriptional increases were observed for *illr1* and *illr3* after infection. A putative binding site for the transcription factor CAAT enhancer-binding protein β (C/EBPβ) in the 5' regulatory region of *illr4* may mediate the transcriptional response

of this gene to infection. These data suggest that zebrafish Illr4 plays an important role in the immune response within zebrafish myeloid cells.

Key words: C-type Lectin, Innate immunity, C/EBPβ, Zebrafish

Abbreviations: ILLR, immune-related, lectin-like receptor; Mincle, macrophage-inducible C-type lectin; DC-SIGN, dendritic cell-specific ICAM grabbing non-integrin; C/EBP, CAAT enhancer-binding protein; SHRV, Snakehead Rhabdovirus; RT-PCR, reverse transcriptase – polymerase chain reaction; qRT-PCR, quantitative RT-PCR.

Introduction

Proteins that possess C-type lectin domains (CTLDs) can be classified into 17 groups based on overall protein structure and domain organization (Drickamer and Fadden 2002; Zelensky and Gready 2005). Group II and Group V CTLD proteins are type II transmembrane proteins with single extracellular CTLD domains and many of these receptors have been associated with immune function. Group II CTLD proteins include dendritic cell-specific ICAM grabbing non-integrin (DC-SIGN/CD209) and multiple DC-SIGN related proteins, macrophage-inducible C-type lectin (Mincle/Clec4e/Clecsf9) and multiple Mincle related proteins, Kuppfer cell receptors and scavenger receptors with a CTLD (SRCL). Group V CTLD proteins include natural killer cell receptors (NKRs) such as the NKG2 and Ly49 families. The ligands for Group II and Group V CTLD receptors typically are carbohydrates and proteins, respectively (Zelensky and Gready 2005)(8).

Immune-related, lectin-like receptor (ILLR) genes (*illr1*, *illr3, illr4/illrL* and the predicted pseudogene *illr2*) were identified from the zebrafish genome in a search for Group V CTLD receptors (Panagos et al. 2006). Although similar to both Group V and Group II CTLD proteins, the ILLRs are phylogenetically classified as Group II CTLD proteins. In addition, zebrafish Illr3 and Illr4 are both catalogued as members of the "CLECT_DC-SIGN_like" sub-family of CTLD proteins by the Conserved Domain Database (CDD) (Marchler-Bauer et al. 2009).

The ILLR genes are encoded on zebrafish chromosome 19: *illr1* and *illr3* are adjacent genes and *illr4* is located ~ 50 Mbp from *illr1* and *illr3*. The ILLR genes

display variable expression within the lymphoid and myeloid lineages: *illr1* transcripts are detected in the lymphoid lineage, *illr3* transcripts are detected in both the lymphoid and myeloid lineage and *illr4* transcripts are detected in the myeloid lineage (Panagos et al. 2006).

The *Mincle* gene was originally shown to be expressed at very low levels in murine macrophages, but displayed a dramatic increase in expression after stimulation with LPS: this increase in *Mincle* expression was then shown to be mediated by the transcription factor, CAAT enhancer-binding protein β (C/EBPβ, also known as NF-IL6) (Matsumoto et al. 1999). As the zebrafish ILLR genes are also expressed at low levels (Yang and Yoder, unpublished observations) it was hypothesized that one or more of the ILLRs may be transcriptionally responsive to immune stimulation. Therefore, adult zebrafish were infected with bacterial or viral pathogens and the relative expression levels of the ILLR genes monitored in multiple tissues. Here we report that the transcription of *illr4* in the spleen is highly responsive to immune challenge, increasing in response to both pathogens. We further show that the C/EBPβ is a likely candidate for regulating *illr4* expression in myeloid cells in zebrafish.

Materials and methods

Animals and tissue sampling

Adult zebrafish were maintained at 28 °C in a recirculating aquarium facility (Aquatic Habitats, Apopka, FL) and fed twice daily. For removal of organs, zebrafish were euthanized in a buffered solution of 0.02% Tricaine methanesulfonate (Finquel MS-222; Argent Chemical Laboratories, Redmond WA).

Streptococcus iniae infections

Adult zebrafish were anesthetized with 0.016% tricaine and either injected intramuscularly with 10 µl 1 x 10^8 cfu/ml *S. iniae*, mock injected with media (THY + P) or not treated as described (Neely et al. 2002). Zebrafish were maintained at 28 °C and euthanized for dissection at 1, 2, 4, 8, 12, 24, and 48 hpi. Tissues were pooled for from 10 zebrafish per treatment and time point for RNA extraction.

Snakehead rhadbovirus *infections*

Adult zebrafish were anesthetized as above and either injected intraperitoneally with 10^5 $TCID_{50}$ (Tissue Culture Infectious Dose) of SHRV/ml, mock injected with PBS or not treated as described (Phelan et al. 2005). Zebrafish were maintained at 28 °C and euthanized for dissection at 2, 4, 8, 12, 24, and 48 hpi. Tissues were pooled for from 10 zebrafish per treatment and time point for RNA extraction.

RNA extraction and cDNA synthesis

Spleens, kidneys, and intestines from euthanized zebrafish were collected directly in TRIzol® (Invitrogen, Carlsbad, CA) and stored at -80 °C. Myeloid and lymphoid cells were FACS sorted (Traver et al. 2003; Trede et al. 2004) into TRIzol and stored at -80 °C. Samples were thawed and homogenized with a Kontes Pellet Pestle® Micro Grinder and RNA purified from TRIzol as directed by the manufacturer. The quality and quantity of the resulting RNA was assessed using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE) and an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA.) Twenty µl cDNA syntheses were performed using total RNA from tissues (2 µg) or cells (1 µg), oligo dT primers and SuperScript III reverse transcriptase as described by the manufacturer (Invitrogen). Reactions were diluted five-fold with RNase-free water.

Quantitative Reverse Transcriptase – Polymerase Chain Reaction (qRT-PCR)

Gene specific primers were designed to detect expression of*, illr1, illr3, illr4, C/EBP*β and *β-actin* (Table 1). Primer pairs span 100-200 bp and at least one intron. All PCRs were performed on an iCycler iQ Detection system (Bio-Rad laboratories, Hercules, CA) in 25 µl volumes comprised of 1 µl cDNA, 1 µl 100 ng/µl forward primer, 1 µl 100 ng/µl reverse primer, and 12.5 µl iQ SYBR Green Supermix (Bio-Rad). Thermal cycling parameters included an initial denaturing at 94 ^{0}C for 2 min, followed by 50 cycles of denaturing at 94 ^{0}C for 30 s, annealing at 60 ^{0}C for 30 s (*β-actin*), or 70 ^{0}C for 30 s (*illr4*), or 65^{0}C for 30 s (*illr1, illr3*, *C/EBP*β), and extension at

72 ^{0}C for 30 s. Reactions were completed in triplicate and the average relative levels of expression were calculated by normalizing to *β-actin* as described (Livak and Schmittgen 2001). Relative values were calculated for infected samples (infected samples compared to untreated samples) and for mock treated samples (media or PBS injected samples to untreated samples). A negative control (no cDNA) for each primer set was used to exclude sample contamination.

Computational analysis

The transcription initiation start sites for the ILLR genes were defined by 5'-RACE PCR (Panagos et al. 2006) and the putative promoter region identified from zebrafish BAC clones CH211-133N4 (*illr1* and *illr3*) and CH211-173P18 (*illr4*). The locations of potential transcription factor binding sites were determined for each ILLR gene using TFSEARCH 1.3 software (http://www.cbrc.jp/research/db/TFSEARCH.html) (Heinemeyer et al. 1998). Searches were performed in the vertebrate category with the threshold score at 85.

Reverse transcriptase PCR (RT-PCR)

Primers were designed to amplify a 780 bp fragment of C/EBPβ by RT-PCR (Table 1). All PCRs were performed in 50 µl volumes comprised of one µl of cDNA, *C/EBPb* forward primer (TCCATGGAAGTGGCCGGTTT), *C/EBP*β reverse primer (CAGTTCTCGTGACAGCTGCT), and Titanium Taq DNA polymerase (Clontech, Mountain View, CA). Thermal cycling parameters included an initial denaturing at 94

^{0}C for 2 min, followed by 35 cycles of denaturing at 94 ^{0}C for 30 s, annealing at 55 ^{0}C for 30 s and extension at 72 ^{0}C for 30 s. PCR primers and conditions for detecting *β-actin*, myeloid-specific peroxidase (*mpx*) and TCRα transcripts by RT-PCR have been described (Panagos et al. 2006).

Results and Discussion

Expression of illr4 increases dramatically after S. iniae infection.

In order to determine if the ILLR genes are transcriptionally responsive to infection, adult zebrafish were immune challenged with *S. iniae* (Neely et al. 2002) and the expression levels of *illr1, illr3,* and *illr4* in the spleen, kidney and intestine were monitored by qRT-PCR. The expression level of *illr1* does not increase in any of the evaluated tissues after *S. iniae* infection (Figure 1a). Although the expression level of *illr3* does not increase in the spleen or kidney after *S. iniae* infection, a slight increase in expression (~5-fold) is detected for *illr3* in the intestine four hours post infection (hpi) (Figure 1b). Interestingly, the expression level of *illr4* in the spleen dramatically increases (~60-fold) 8 hpi with *S. iniae* followed by a decline in expression through 48 hpi. No major increase in *illr4* expression is detected in the kidney after *S. iniae* infection and only a slight, but maintained increase (~5 to 8-fold) of *illr4* expression is detected in the intestine after *S. iniae* infection (Figure 1c). Overall, the most dramatic change in ILLR expression after *S. iniae* infection was observed for *illr4* in the spleen at 8 hpi.

Expression of illr4 increases dramatically after SHRV infection.

In order to determine if the transcriptional response of *illr4* to *S. iniae* was specific to that pathogen, the expression of the ILLR genes was monitored in the spleen, kidney and intestine after immune challenge with SHRV (Phelan et al. 2005). The expression of *illr1* and *illr3* in all tissues evaluated did not increase after SHRV infection (Figure 2a-b). However, as with the *S. iniae* infection, *illr4* expression started to increase at 8 hpi (~15-fold) and dramatically increased (~30-fold) at 12 hpi followed by a decline (~10-fold) at 24 hpi (Figure 2c). It is noted that the injection of PBS induced *illr3* expression (~12-fold) in both the spleen and intestine at 2 hpi, however, this increase was not observed for the injected SHRV and not observed for the PBS injected samples in a biological replicate (data not shown). The quality of purified RNA at 48 hpi SHRV was relatively poor (likely due to necrosis) and thus excluded from analyses. Overall, the most dramatic change in ILLR expression after SHRV infection was observed for *illr4* in the spleen at 12 hpi.

The proximal promoter region of illr4 includes a putative C/EBPβ binding site.

As transcription of *illr4* was highly responsive to infection, we considered what transcription factors might play a role in regulating *illr4*. We previously reported that the Illr4 protein sequence is similar to two salmon C-type lectin receptors, SCLRA (also named CD209 antigen-like protein E) and SCLRB (also named C-type lectin domain family 4 member E) (Panagos et al. 2006). SCLRA and SCLRB have been shown to increase in expression in response to *in vivo* infection (*Aeromonas*

salmoncida) and in response to LPS stimulation in cell culture (Soanes et al. 2004). Putative binding sites for the transcription factor, C/EBPβ, were identified in the proximal promoter regions of SCLRA and SCLRB and it was suggested that C/EBPβ may play a role in the transcriptional regulation of these salmon C-type lectin receptors (Soanes et al. 2004). This suggestion was founded on the observation that C/EBPβ is a major regulator of Mincle expression in stimulated mouse macrophages (Matsumoto et al. 1999). Approximately 2.0 kb of each putative promoter region from the zebrafish *illr1*, *illr3* and *illr4* genes were scanned (TFSEARCH 1.3) for potential C/EBP transcription factor binding sites. Although C/EBP binding sites were identified in the promoter regions for all three ILLR genes, only *illr4* included a C/EBPβ binding site within 50 bp of the predicted transcriptional start site (Figure 3a).

C/EBPβ is expressed in hematopoietic lineages.

The functional motifs of C/EBPβ are well conserved between zebrafish and human (Lyons et al. 2001a; Lyons et al. 2001b). Although zebrafish C/EBPβ has been shown to be expressed in embryonic liver, gut, and macrophages (Lyons et al. 2001a; Lyons et al. 2001b; Thisse 2001), it has not been determined if C/EBPβ is expressed in adult tissues and hematopoietic lineages.

We previously reported that the *illr4* gene is expressed in the myeloid lineage but not in the lymphoid lineage of zebrafish (Panagos et al. 2006). In order for C/EBPβ to influence *illr4* expression in myeloid cells, it must be expressed in the myeloid lineage. Therefore, the expression of C/EBPβ in zebrafish liver, kidney,

spleen and intestine as well as FACS-sorted lymphoid and myeloid cells was evaluated by RT-PCR and C/EBPβ expression was detected in all tissues and hematopoietic cells evaluated (Figure 3b).

Expression of C/EBPβ increases after S. iniae infection.

In order to evaluate the potential of C/EBPβ to influence *illr4* expression, the expression level of C/EBPβ was evaluated in the spleen after *S. iniae* infection (Figure 3c). At 1 hpi an immediate increase (~10-fold) in *C/EBPβ* expression was observed which fell by 2 hpi. *C/EBPβ* transcript levels in the spleen stay slightly above the levels in mock infected zebrafish until 12 hpi when *C/EBPβ* expression increase slightly (~5-fold). These observations are consistent with other studies demonstrating *C/EBPβ* expression drastically increases in mouse lung, liver, kidney and spleen 4 hr after injection with LPS (Akira et al. 1990) and was shown to increase ~30-fold in individual adult zebrafish at 2 hpi with *Listonella anguillarum* (Rojo et al. 2007). The early transcriptional response of *C/EBPβ* to *S. iniae* infection and the later transcriptional response of *illr4* to *S. iniae* infection are consistent with the hypothesis that C/EBPβ can directly regulate *illr4* expression.

Summary

Here we demonstrate that *illr4* (but not *illr1* and *illr3*) expression is drastically increased in the zebrafish spleen approximately 8-12 hpi with both bacterial and viral pathogens. We further show that the *illr4* promoter possesses a putative C/EBPβ

binding site and that *C/EBPβ* expression increases as early as 1 hpi supporting the hypothesis that C/EBPβ regulates *illr4* expression.

These observations lead to questioning the functional role of Illr4 in the zebrafish immune response. Illr4 is expressed in myeloid cells and based on the presence of a putative cytoplasmic immunoreceptor tyrosine-based inhibition motif (ITIM) has been predicted to function in an inhibitory pathway (Panagos et al. 2006). However, the mannose receptor (a Group VI CTLD protein) possesses a cytoplasmic tyrosine that has been implicated in receptor mediated phagocytosis, endocytosis and endosomal sorting (Schweizer et al. 2000). In addition, Illr4 possesses a cytoplasmic penta-acidic sequence (DDEDD) which is not present in Illr1 or Illr3. A cytoplasmic tri-acidic sequence in other Group II CTLD receptors (e.g. DC-SIGN) is required for efficient cell surface expression and for lysosomal targeting during endocytosis (Azad et al. 2008). The observation that illr4 expression is drastically increased after both bacterial and viral infection indicates that it plays in important role in the response to or recovery from infection: whether Illr4 functions to initiate an inhibitory signal within myeloid cells or to endocytose pathogens remains to be determined.

Acknowledgments

The authors wish to acknowledge the skillful technical assistance of Radhika Shah and Tim Orcutt. This work was supported by a grant from the NC State University College of Veterinary Medicine.

Table 1. Sequence information for qRT-PCR[1] primers

Gene	Primer name	Primer sequence
illr1	Illr1_RT_F	AGTGGCAAATGCTCCCAATAC
	Illr1_RT_R	GCAACGACAAGACAAACGATG
illr3	Illr3_RT_F	CTGTGTTCGCCTTTTCAACA
	Illr3_RT_R	CTGCGCTGAAACACTGACAT
illrL	IllrL_RT_F	AAGAAACAGGCGGACAAAGA
	IllrL_RT_R	ATCACACACAGAGCCGTCAG
C/EBP β	C/EBPb_RT_F	CGTACCTGCCGTACCAGACT
	C/EBPb_RT_R	TGTACTCGTCGCTGTCCTTG
β-actin	bactin_RT_F	AGAGCTACGAGCTGCCTGAC
	bactin_RT_R	TCTCGTGGATACCGCAAGAC

[1] qRT-PCR = Quantitative RT-PCR

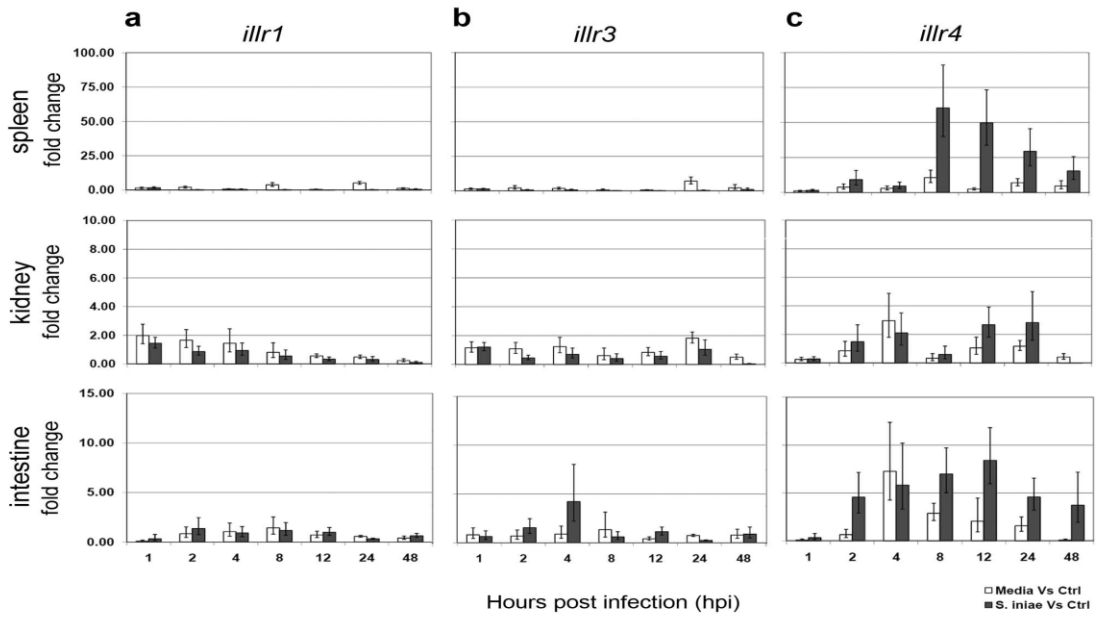


Figure 1. Quantitative RT-PCR (qRT-PCR) analysis of ILLR gene expression in *S. iniae* infected zebrafish tissues. Adult zebrafish were injected with *S. iniae*, media only or not treated as a control reference (Ctrl). qRT-PCR was used to evaluate the relative expression levels of *illr1* (**a**), *illr3* (**b**), and *illr4* (**c**) in the spleen, kidney and intestine at 1, 2, 4, 8, 12, 24, and 48 hpi (X axis). Values were normalized to β actin and the relative fold-difference in expression was calculated ($2^{-\Delta\Delta CT}$) as described (105). Values (Y axis) represent normalized expression levels in infected (grey bars) or media injected (white bars) zebrafish relative to expression in control zebrafish. Error bars represent standard deviation. Data shown are representative of two biological replicate studies.

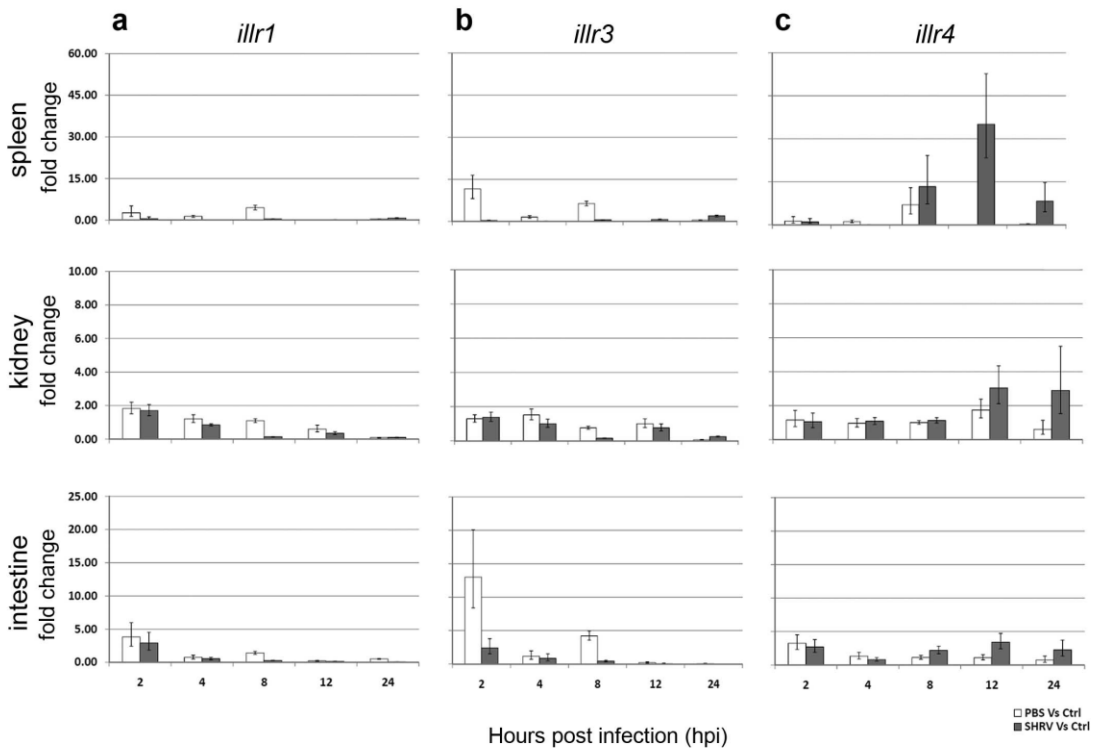
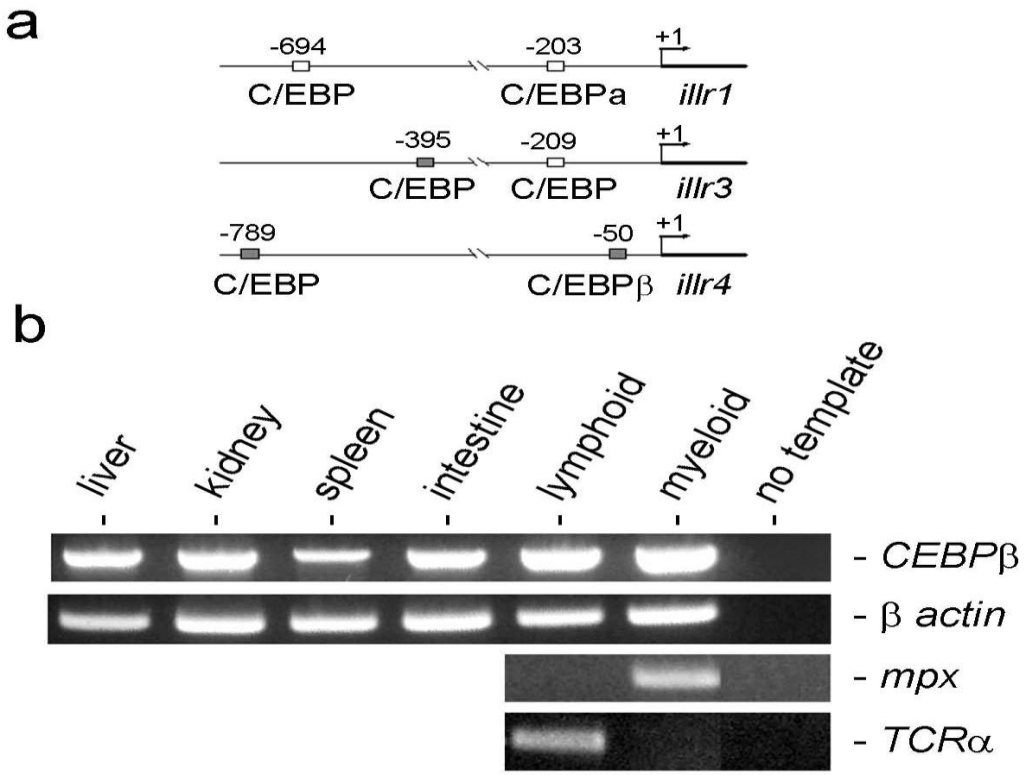


Figure 2. Quantitative RT-PCR (qRT-PCR) analysis of ILLR gene expression in *SHRV* infected zebrafish tissues. Adult zebrafish were injected with *SHRV*, PBS only or not treated as a control reference (Ctrl). qRT-PCR was used to evaluate the relative expression levels of *illr1* (**a**), *illr3* (**b**), and *illr4* (**c**) in the spleen, kidney and intestine at 2, 4, 8, 12, and 24 hpi (X axis). Values were normalized to β actin and the relative fold-difference in expression was calculated ($2^{-\Delta\Delta CT}$) as described (105). Values (Y axis) were calculated as in Fig 1. Data shown are representative of two biological replicate studies.

Figure 3. C/EBPβ may regulate *illr4* expression. **a** All ILLR genes possess putative C/EBP binding sites. Only *illr4* possesses a candidate C/EBPβ binding site. The +1 above each arrow indicates the predicated transcriptional start site. White and grey boxes represent potential transcription factor binding sites (TFBSs) identified on the – and + strands, respectively. Numbers above boxes indicate the positions of the TFBSs relative to the transcriptional start site. **b** Full-length CEBPβ transcripts were detected by non-quantitative RT-PCR from adult zebrafish liver, kidney, spleen, intestine, and FACS-sorted lymphoid and myeloid cells. Analysis of *β-actin* is included as a positive control. Analyses of *mpx* and *TCR*α are included as controls for the myeloid and lymphoid cell RNAs, respectively. **c** qRT-PCR was performed using splenic RNA from *S. iniae* infected zebrafish at 1, 2, 4, 8, 12, 24, and 48 hpi (X axis). Values (Y axis) were calculated as in Fig 1.


a
-694
-203
+1
C/EBP
C/EBPa
illr1
-395
-209
+1
C/EBP
C/EBP
illr3
-789
-50
+1
C/EBP
C/EBPβ
illr4
b
liver
kidney
spleen
intestine
lymphoid
myeloid
no template
- CEBPβ
- β actin
- mpx
- TCRα
c
15.00
10.00
5.00
0.00
Fold change
Media Vs Ctrl
S. iniae Vs Ctrl
1
2
4
8
12
24
48
Hours post infection (hpi)

References:

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CHAPTER 4. EVALUATION OF *illrL* EXPRESSION IN *S. iniae* INFECTED TRANGENIC ZEBRAFISH

Abstract

Results shown in Chapter III demonstrate elevated transcription of *illrL* after immune challenge using the pathogens *S. iniae* and Snakehead rhabdovirus to infect zebrafish. In order to determine if *S. iniae* infection could help identify *illrL* positive cells (via increased *illrL* transcription), *illrL*^{2k}*:mCherry* transgenic fish were infected with this pathogen and their splenocytes evaluated for mCherry expression by flow cytometry. Although an increase in mCherry intensity was detected in the transgenic splenocytes, it was not at a significant level (p = 0.07). These data indicate that *illrL* is expressed at an extremely low level. This is consistent with our results employing RISH and transgenic strategies to detect ILLR gene expression in untreated (not infected) zebrafish (Chapter II). Strategies such as monoclonal antibody development may be helpful to identify, purify, and characterize IllrL expressing cells in zebrafish.

Introduction

Two decades ago, zebrafish was established as a basic model system to study vertebrate development and genetics. Recent advances in genomic resources reveal that zebrafish share many orthologous immune-related genes with mouse and human (1, 2). With these advantages, the zebrafish has become an alternative vertebrate model system for evaluating gene function under healthy and diseased conditions. *S. iniae* is a natural fish pathogen that can be transpassed to humans during the process of handling infected fish (3, 4). Neely et al. reported that the zebrafish model for Streptococcal *(S. iniae)* infection displayed comparable changes in clinical signs and histopathology as in Streptococcal infections in mice and humans (6, 7). These observations support the use of zebrafish as a model to study host-pathogen interactions for human diseases. Zebrafish IllrL is a C-type lectin-like receptor and may share the typical functions as its mammalian counterparts, such as antigen recognition. The question we are interested to ask here is whether *illrL* expression increases after *S. iniae* infection in *illrL* transgenic fish. In this regard, we infected adult zebrafish with *S. iniae*, resulting in the dramatic induction of *illrL* in spleen cells. Enlarged spleen tissue in immune challenged fish indicated an acute infection. This is the first preliminary study to describe *illrL* as a gene involved in zebrafish innate immunity.

Materials and Methods

Animals. Adult AB wild type zebrafish and transgenic fish (*illrL*^{2k}*:mCherry*) were maintained as described in Chapter 2.

Bacterial strain, media, and growth condition. The streptococcal strain *S. iniae* 9117 was originally obtained from the blood of a clinical patient of cellulitis and shipped to our lab from Dr. Melody Neely's lab (Wayne State University College of Medicine). Overnight cultures of *S. iniae* were in TP media (ThyB plus peptone media, to make TP media, dissolve 30 g Todd-Hewitt, 1 g Yeast extract, and 20 g Proteose Peptone in 1L ddH2O). A 1:100 dilution of overnight culture was made in fresh TP media and grown for approximately 3 hours so that the final OD_{600} was between 0.225 to 0.25. One ml of culture was centrifuged at 13,000 rpm for 3 min, and then resuspended in 1 ml fresh TP media. Enumeration of bacterial cultures were determined by serial dilution on ThyA agar plates (to make ThyA agar plates, dissolve 30 g Todd-Hewitt, 1 g Yeast extract, 20 g Proteose Peptone, and 14g bacto agar in 1L ddH2O).

Experimental infection. Infection of zebrafish with *S. iniae* was performed as described in Chapter 3 (5).

Quantitative real-time PCR. RNA isolation and cDNA synthesis were described in Chapter 2. Real-time PCR was performed as described in Chapter 2.

Flow cytometry. Cell collection and flow cytometry were performed on splenocytes from infected wild type and founder transgenic fish (*illrL*^{2k}*:mCherry*) as described in Chapter 2 (11).

Results and Discussion

IllrL expression increased after *S. iniae* infection

In order to confirm that *S. iniae* infections performed in our laboratory can induce an increase in *illrL* expression, qRT-PCR was used to analyze *illrL* expression at 8 hpi with *S. iniae* in wild type fish spleen tissue. This replication study indicated that *illrL* expression was over 100 fold higher in infected relative to uninfected fish (Fig. 1), which is comparable with the previous result when infections were completed in Dr. Neely's laboratory (approximately 60-fold change). In addition, *S. iniae* infection caused splenomegly at 12 hpi (Fig. 2). This observation was not unexpected as the spleen is a major peripheral lymphoid site. Spleen enlargment may indicate a migration of immune cells such as macrophages and DC to spleen. Or the spleen could undergo hypertrophy. Histopathologic examination of spleen tissue will provide further information about the cellular components in the inflammatory response.

Flow cytometric analysis on infected transgenic fish (*illrL*^{2k}*:mCherry*)

In order to determine how many zebrafish spleens would be required to collect enough cells (10,000 events per sample) for analysis by flow cytometry, spleens from one, three or six infected wild type zebrafish were pooled. Using pooled control (not infected) splenocytes

from 1, 3 and 6 wild type zebrafish background fluorescence was observed in 0.49%, 1.92%, and 4.14% of the splenocytes, respectively (Table 1). This suggests a trend that a higher percentage of mCherry background positive cells appear if there are cells from several fish pooled together. However, we did not see this trend when cells of infected wild type zebrafish were pooled (background fluorescence of 1.89%, 1.73% and 0.64% in cells from 1, 3, and 6 zebrafish respectively). Because one dissected spleen will generate enough cells for flow cytomytry, future studies did not employ cells pooled from several fish.

Wild type and transgenic fish (*illrL*^{2k}*:mCherry*) were then infected with *S. iniae* and spleens were dissected at 12 hpi. Spleens from individual fish were used to make single cell suspensions. The results shown in Fig 3A from infected transgenic fish showed a slight increase in the percentage of mCherry positive cells (1.3 ± 0.54%, mean ± standard deviation, $p = 0.5$, n = 3) compared to wild type control group (0.96 ± 0.23%, n = 3). As shown in Fig 3B, infected transgenic fish express slightly more mCherry as indicated by mean fluorescence intensity (70 ± 7 vs. 54.4 ± 3). This suggests that immune challenge with *S. iniae* increases mCherry expression at the protein level, but the difference does not reach a statistically significant level ($p = 0.07$ when measured by MFI). Further studies using *S. iniae* infected transgenic fish (*illrL*^{2k}*:mCherry*) are required before a definitive conclusion about *illrL* expression after infection can be reached.

Our previous study (Chapter 3) showed an approximately 60-fold increase of *illrL* at the transcription level in *S. iniae* infected zebrafish as compared to uninfected control zebrafish.

The different results between this previous study and our flow cytometry data using infected *illrL*^{2k}*:mCherry* zebrafish is inconsistent, but can be explained by several reasons. First, we measured changes in *illrL* transcription by real-time PCR which is likely more sensitive than flow cytometry to detect mCherry protein. mCherry is photo-sensitive with a half-life less than five minutes, the shortest one among all the common fluorescent proteins (12). Although very careful steps were taken to prevent light exposure, it is possible that the cells were exposed to light during the treatment procedures. Second, the 2 kb promoter region of *illrL* used to drive mCherry expression may not include the entire transcriptional regulation sequence. Even though GFP+ hearts were detected in the *illrL*^{2k}*:mCherry* zebrafish, this only validated that the transgene was present, not that the illrL promoter was functional. In order to test this possibility, one could quantify *mCherry* expression transcripts at 8 or 12 hpi by real-time PCR on infected *illrL*^{2k}*:mCherry* zebrafish. If *mCherry* is shown to increase at the transcriptional level, it would demonstrate that the 2 kb *illrL* promoter region at least includes the immune mediated regulatory elements. Third, *mCherry* transcripts may not be efficiently translated at the time point after infection when fish were euthanized. This may be caused by evaluating mCherry expression at incorrect time periods or by endogenous posttranscriptional regulatory networks. The *illrL* transcripts reached the highest level at 8 hpi among the analyzed time points, and we dissected the infected *illrL*^{2k}*:mCherry* zebrafish at 12 hpi, providing a 4-hour delay to permit mCherry translation. Was 4 hours enough time for *mCherry* transcripts to be translated? Evaluating mCherry expression at later time points could help to answer this question. Posttranscriptional regulatory networks control gene expression by regulating mRNA stability, translation and pre-mRNA processing (13). Since

mCherry is not a gene of zebrafish, perhaps zebrafish do not have the proteins necessary to process the transcripts for proper posttranscriptional or posttranslational modifications. However, many other laboratories have successfully used mCherry in zebrafish transgenic lines. In human NK cells, the cell surface expression of KIR proteins accurately corresponds with the presence of KIR mRNA (14). This indicates that human KIR expression is regulated at the transcriptional level. Last, the up-regulated level of *illrL* was lower than *illr1* and *illr3* based on the Ct values (Figure 1 in Appendix). Ct means the number of threshold cycles where the amount of PCR product reaches the baseline level. It suggests that *illrL* could be expressed at the lowest level among the gene family if we assume that all sets of primers work at the same efficiency.

Overall, this infection study of transgenic fish further reveals that ILLRs could be a family of CLRs in zebrafish with extremely low level expression, or that the transgenes did not have the correct promoter sequence. A better understanding of the molecular regulation mechanisms of *illrL* gene expression by promoter analysis may help characterize *illrL* expression in this immune model.

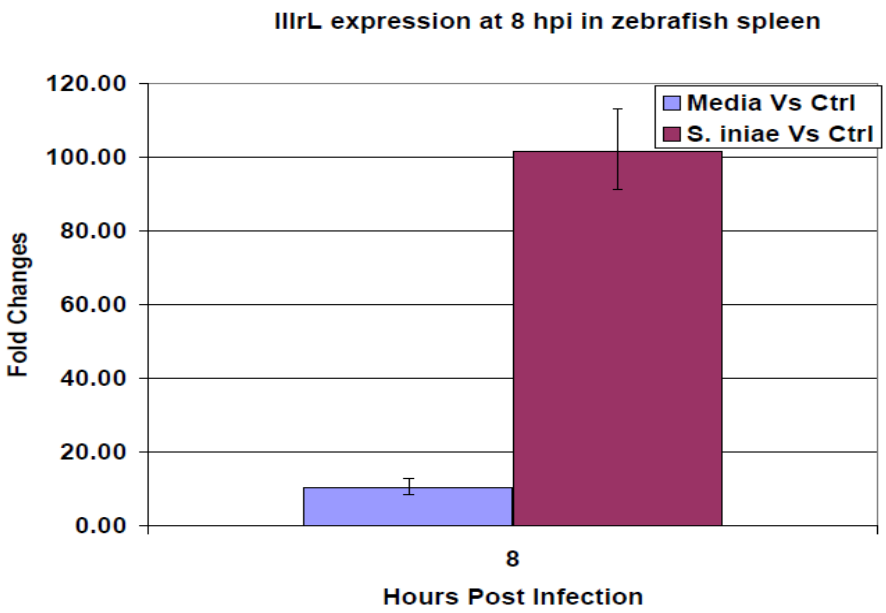


Figure 1. Real-Time PCR analysis of *illrL* expression after *S. iniae* infection. Wild type zebrafish were divided into three groups (10 fish per group) including 1) uninjected, 2) injected with TP media, and 3) injected intramuscularly with 10 µl of 1 X 10^8 cfu/ml *S. iniae* culture. Spleens were dissected at 8 hpi and RNA was recovered, and reverse transcribed. Real-Time PCR analysis demonstrated that *illrL* transcript levels increased approximately 100-fold following *S. iniae* infection (relative to uninjected fish). This transcriptional response is specific for the pathogen as illrL transcript levels increase only ~ 10-fold following the injection of media alone (relative to uninjected zebrafish). Data shown are one representative study performed in our own lab. Each bar represents the mean of three replicates. Error bars represent standard deviation.

Figure 2. Zebrafish spleen following infection of *S. iniae*. Appearance of the spleen tissue from one of the transgenic fish at 12 hpi.

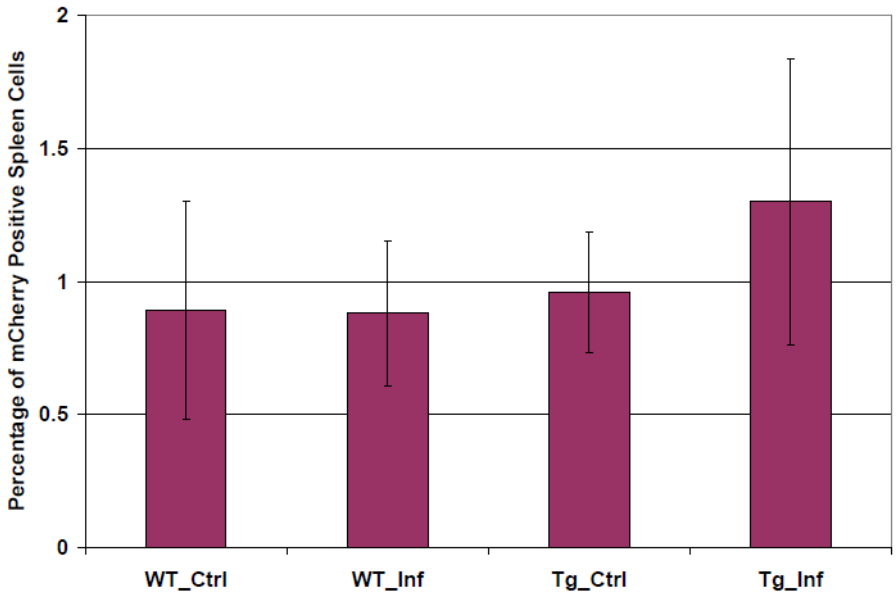
Table 1. FACS analysis of spleen cells representing mCherry background in wild type zebrafish.

Sample #	Sample ID*	Percentage of mCherry background positive cells in total spleen cells (%)	Mean Fluorescence Intensity (MFI)
1	WT_Ctrl_1	0.49	78.97
2	WT_Ctrl_3	1.92	69.37
3	WT_Ctrl_6	4.14	81.58
4	WT_Inf_1	1.89	60.52
5	WT_Inf_3	1.73	60.09
6	WT_Inf_6	0.64	69.42

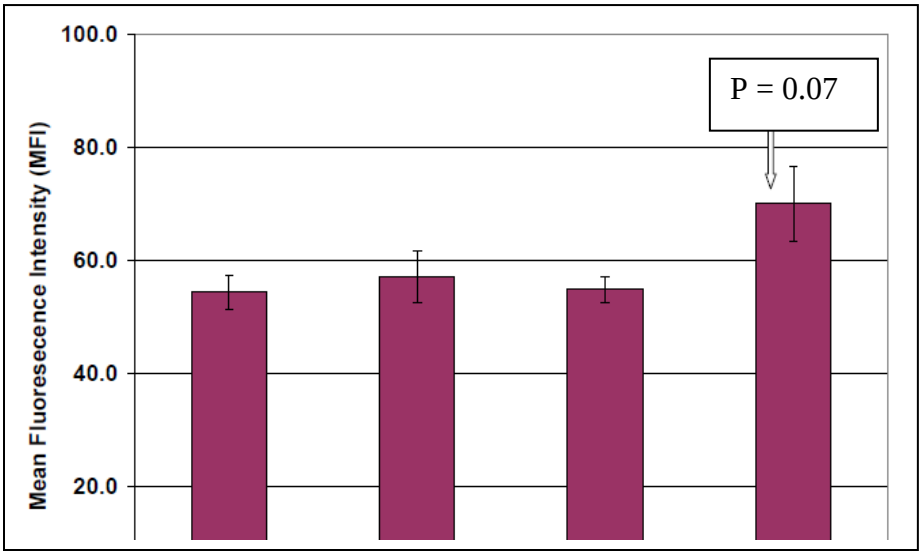
* = the last number in the ID name indicates how many fish are grouped together in order to collect enough spleen cells for FACS analysis.

Figure 3. FACS analysis of mCherry positive spleen cells in transgenic zebrafish. Either wild type or *illrL*^{2k}*:mCherry* zebrafish were injected intramuscularly with 10 µl of 1 X 10^8 cfu/ml *S. iniae* culture. At 12 hpi, three individual fish in each group (wild type uninjected control, wild type infected, transgenic uninjected control, and transgenic infected groups) were euthanized and spleen tissues were isolated to make single cell suspensions. Cells were analyzed by a flow cytometry. (**A**) The percentage of mCherry positive splenocytes is shown. (**B**) The mean fluorescence intensity (MFI) in the corresponding groups from (A) is shown. Student T- test statistical analysis was used to analyze the data. Each bar represents the mean of three replicates. Error bars represent standard deviation.

A.


Percentage of mCherry Positive Spleen Cells
2
1.5
1
0.5
0
WT_Ctrl
WT_Inf
Tg_Ctrl
Tg_Inf

B.


Mean Fluoresecence Intensity (MFI)
100.0
80.0
60.0
40.0
20.0
P = 0.07

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CHAPTER 5. CLONING ZEBRAFISH IMMUNE-RELATED LECTIN-LIKE RECEPTOR 5

Abstract

A family of immune-related, lectin-like receptor (ILLR) genes have been characterized on zebrafish (*Danio rerio*) chromosome 19 and classified as Group II C-type lectin-like domain (CTLD) receptors (*illr1*, predicted pseudogene *illr2*, *illr3* and *illrL*). Herein, we describe a new member of the ILLR family *illr5* which is located on chromosome 19 in a gene cluster with *illr1* and *illr3*. An *illr5* cDNA sequence (867 bp) which was cloned by rapid amplification of cDNA ends (RACE) encodes 289 amino acids. Illr5 is a Group II CTLD receptor that possesses a cytoplasmic ITIM-like motif and a single extracellular carbohydrate-recognition lectin-like domain (CRLD) of approximately 136 residues. Phylogenetic analyse confirms that Illr5 belongs to the ILLR family. Reverse transcription PCR analyses demonstrate that *illr5* is expressed in the lymphoid lineage, not in myeloid lineage, suggesting that Illr5 may play an important role in the immune response of hematopoietic cell lineages.

Introduction

Lectins are defined as proteins able to recognize and bind carbohydrates. Animals contain a variety of lectins involved in either cell-cell interactions during development and differentiation or the recognition of foreign molecules from pathogens (1). C-type lectin receptors represent a large superfamily of lectins that recognize carbohydrates through a highly folded carbohydrate recognition domain (CRD) in a calcium-dependent manner. Based on protein architecture, the family of proteins encoding C-type lectin domains has been classified into 17 groups (Groups I, II, III,…XVII) (2). In humans, the C-type lectin NKR family is encoded in a single chromosomal region 12p12-p13 (3, 4). Group V CTLD proteins include the mammalian NK cell receptor (NKR) families Ly49 and NKG2 which contain intracellular inhibitory or activating signaling domains (5, 6). A similar region encoding the Ly49 gene family is present on mouse chromosome 6. Genes encoding putative C-type lectin NKRs have also been revealed in some teleostean fishes such as rainbow trout (*Oncorhynchus mykiss*), carp (*Cyprinus carpio*), eel (*Anguilla japonica*), salmon (*Salmo salar*), and Japanese flounder (*Paralichthys olivaceus*) (7-11).

One family of immune-related lectin-like receptor (ILLR) genes has been characterized on zebrafish (*Danio rerio*) chromosome 19 (12). They are classified as Group II C-type lectin domain (CTLD) receptors (e.g. *illr1*, predicted psuedogene *illr2*, *illr3* and *illrL*). The ILLR genes contain inhibitory or activating signaling motifs similar to Group V CTLD NK receptors (13). Here we report characterization of an additional ILLR gene, *illr5*. Transcripts

of *illr5* are detected in lymphoid cells, but not myeloid cells suggesting that Illr5 may function in lymphocytes or NK cells. Similar to *illr1* and *illrL*, *illr5* possesses a cytoplasmic sequence similar to an immunoreceptor tyrosine-based inhibitory motif (ITIM).

Materials and methods

RNA extraction and cDNA synthesis was described in Chapter 3.

Rapid amplification of complementary DNA (cDNA) ends (RACE). RACE-ready cDNA was generated according to the manufacturer's protocol (GeneRacer, Invitrogen). Gene Specific Primers (GSPs) for *illr5* were designed to amplify the predicted *illr5* sequence identified in the zebrafish BAC 211-133n4 (Table 1). Both primary and nested RACE PCR was performed with "touch-down" PCR as previously described (12). All cDNAs were cloned into pGEM-T (Promega) and sequenced.

Phylogenetic tree analysis. Predicted lectin-like domains were identified with the Simple Modular Architecture Research Tool software (http://smart.embl-heidelberg.de/) and aligned using ClustalW (http://www.ch.embnet.org/software/ClustalW.html) (14, 15). The phylogenetic tree was constructed using the aligned CTLDs from Group II and V C-type lectin-like receptors using the neighbor-joining method and 80% bootstrap value within MEGA4.1 software (16, 17). CTLD sequences were derived from the following GenBank accession numbers: zebrafish Illr1, (AY986755); Illr3_a, (AY986756); IllrL, (AY986758);

Oncorhynchus mykiss CD209-like, (AY593994); human killer cell lectin-like receptor subfamily K member 1(KLRK1, NM_007360); MINCLE/CLECSF9, (AY358499); mouse DC-SIGN/CD209, (BC111095); CD209-like, (AY593994); mouse NKG2D, (AF054819); Ly49, (AY003920); C-type lectin domain family 4 member f (Clec4f, NM_016751); asialoglycoprotein receptor 2 (Asgr2, NM_007493); macrophage galactose N-acetyl-galactosamine specific lectin-1 (Mgl1, NM_010796); Mgl2, (NM_145137); salmon C-type lectin receptor A (SCLRA, AY572832); SCLRB, (AY572833), and SCLRC, (AY572834).

Reverse transcriptase PCR (RT-PCR). To analyze expression levels of *illr5* at early stages during development, zebrafish larvae were included at 0, 6, 12, 24, 36, 48, 72 hours, and 6.5 days post fertilization. Myeloid and lymphoid cells from adult zebrafish were purified by cell sorting as described (18, 19). RT-PCR was performed using *illr5* gene specific primers (Table 1) according to the method described in Chapter 3. The PCR reaction was incubated at 94 ^{0}C for 2 min, followed by 38 cycles of 94 ^{0}C for 30 s, 65 ^{0}C for 30 s, and 72 ^{0}C for 30 s. β-actin was amplified with 25 cycles at the annealing temperature 65 ^{0}C.

Results and Discussion

Protein sequence comparison and predicted structures of zebrafish ILLRs

The zebrafish ILLR genes, *illr1*, *illr3* and *illrL/illr4* are present on chromosome 19 and have been previously characterized (12). Here we report an additional member of this family

named *illr5* which is also present on chromosome 19. *Illr5* genomic sequence was obtained directly by searching the zebrafish Zv7 database (http://www.ensembl.org/Danio_rerio/). RACE PCR was employed to amplify its full-length ORF cDNA.The predicted protein sequence of Illr5 was aligned with the previously described ILLRs in Figure 1A. Illr5 is a type II transmembrane receptor with a single CTLD; however, Illr5 does not possess two specific cysteines found in all other ILLRs (indicated by a diamond in CTLD in Fig 1a) which could form additional disulfide bonds in NKRs and other CTLDs (7). Mammalian inhibitory NKRs typically possess one or more ITIMs (S/I/V/LxYxxI/V/L) (21). Illr5 possesses a cytoplasmic ITIM-like sequence (itim, SxYxxS) similar to Illr1 and Illr3. This suggests that Illr5, upon ligand recognition, may initiate an inhibitory cytoplasmic signaling pathway. A previous study by Panagos et al. suggested that *illr2* might be a pseudogene. We have also cloned and sequenced an *illr2* cDNA demonstrating that it encodes a non-functional transcript with two premature stop codes (Appendix Figure 2).

Phylogenetic tree analysis of *illr5* CTLD and its genomic organization

A phylogenetic comparison of the CTLDs of the ILLRs and other lectin-like receptors shows that Illr5 groups with other zebrafish ILLRs demonstrating that all ILLR CTLDs (including Illr5) are more similar to mammalian Group II CTLD proteins than to Group V proteins such as NKG2D and Ly49 (Figure 2). As described previously, *illr1* and *illr3* are encoded in BAC clone CH211-133N4 and *illrL* is encoded in CH211-173P18 (12). A Blastx search (http://blast.ncbi.nlm.nih.gov/Blast.cgi) with *illr5* cDNA allowed us to define its genomic

organization within CH211-133N4 (Figure 3). The *illr5* gene, along with *illr1* and *illr3* define a small ILLR gene cluster; however, *illrL* is encoded at a distant and distinct locus on the same chromosome.

RT-PCR analysis of *illr5* expression pattern

To determine the tissue expression pattern of *illr5*, RT-PCR was performed on zebrafish tissues (liver, kidney, spleen, and intestine) and myeloid and lymphoid cell lineages. The results indicate that *illr5* is expressed primarily in the hematopoietic spleen and kidney, but not in the liver (Fig. 4). Transcripts of *illr5* cannot be detected in zebrafish larvae before 6 days old (data not shown). Transcripts of *illr5* can also be detected in the intestine, but at a lower level than in spleen and kidney. Interestingly, RT-PCR analysis on purified lymphoid and myeloid cells showed that *illr5* was exclusively expressed in the lymphoid lineage, not in the myeloid lineage. As mentioned before, *illr5* contains an ITIM-like sequence in its intracellular domain, which suggests that Illr5 likely functions as an inhibitory receptor on lymphocytes.

In conclusion, this study demonstrates the existence of a new member of the ILLR family, *illr5*, which is encoded on zebrafish chromosome 19. Illr5 is a type II transmembrane lectin-like receptor with an intracellular itim motif. As numerous C-type lectin-like receptors have been shown to play important roles in innate immunity (they recognize pathogen via conserved patterns, directly neutralize antigens, and mediate intercellular adhesion), further

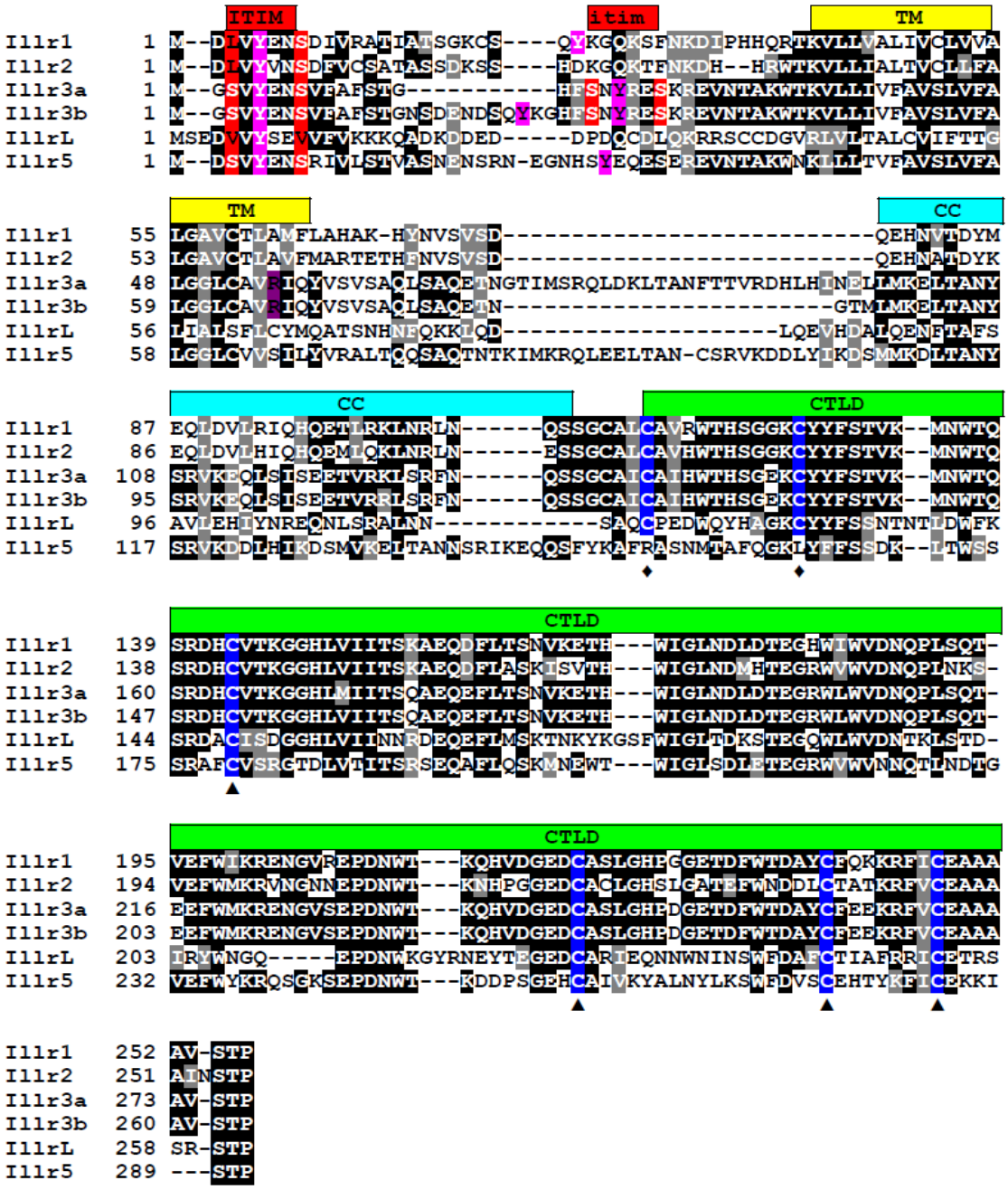
functional characterization of Illr5 at the protein and cellular levels will be necessary to define its role in zebrafish innate immunity.

Table 1. Sequence information for oligonucleotide primers.

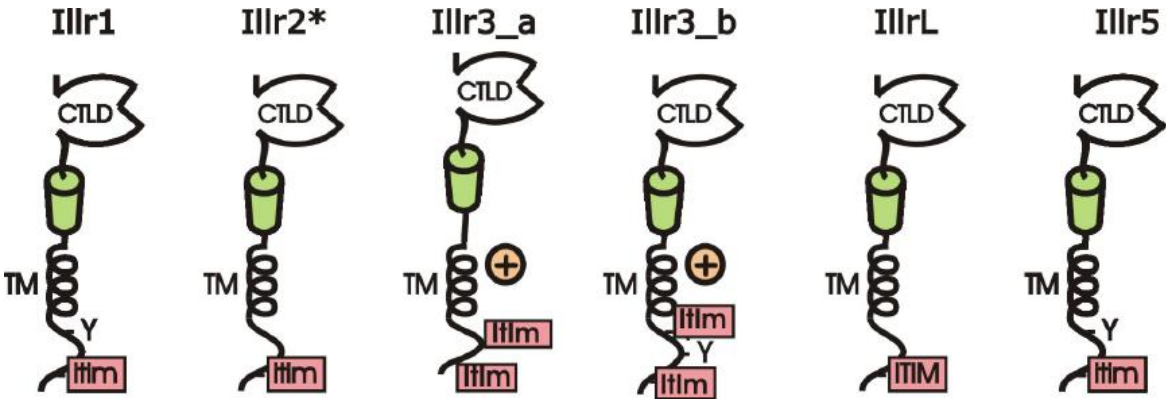
Purpose	*Primer name*	*Primer sequence*
Illr2 3' RACE	Illr2_3'RACE_F1	CAGAAGTAAGATATCCGTCAGGCT
	Illr2_3'RACE_F2	ATGGATCTGGTTTATGTCAATTC
Illr2 5' RACE	Illr2_5'RACE_R1	CAGTTATCAGGTTCATTGTTCCCA
	Illr2_5'RACE_R2	TCCCATTCACTCGTTTCATCCAGA
Illr5 3' RACE	Illr5_3'RACE_F1	CACCATAACCAGCCGATCTGAACA
	Illr5_3'RACE_F2	ACGAATGGACATGGATTGGACTCA
Illr5 5' RACE	Illr5_5'RACE_R1	CTCTGCTGTAGTTCGCTGTCAGAT
	Illr5_5'RACE_R2	CAGTTCCTCAAGCTGTCTCTTCAT
RT-PCR illr5	Illr5 For	ATGGACTCAGTCTATGAAAATTC
	Illr5 Rev	TTCTTTTCGCAGATGAACTTGTA
RT-PCR β-actin	β-actin For	GGTATGGAATCTTGCGGTATCCAC
	β-actin Rev	ATGGGCCAGACTCATCGTACTCCT

Figure 1. The ILLR family. (**A**) Alignment of amino acid sequences of zebrafish ILLRs. The conserved and identical residues are represented by black shading, and grey shading indicates similar amino acids. Transmembrane (TM), coiled coil (CC), and C-type lectin domains (CTLD) are shown above the alignment. IllrL contains a cytoplasmic ITIM, and other ILLRs contain ITIM-like sequences (itim). Additional cytoplasmic tyrosines are highlighted in pink. Illr3a and illr3b include a positive charged residue (Arg in violet color) in their transmembrane domain. Conserved cysteines within the CTLD are highlighted with blue. A diamond indicates cysteines present in NK cell receptors or other CTLDs. An upward arrow-heads show identical cysteines present in all ILLRs. Amino acid numbering is on the left. Gaps in the amino acid sequence alignment are indicated with dashes. (**B**) Predicted protein architecture of ILLRs. ILLRs are type II transmembrane proteins with a single extracellular CTLD and coiled coil domain (green). The asterisk above Illr2 indicates that the structure is predicted from genomic sequence.

A.


ITIM
itim
TM
TM
CC
CC
CTLD
CTLD
CTLD
Il1r1 1 M--DLVYENSDIVRATIATSGKCS----QYKGQKSFNKDIPHHQRTKVLLVALIVCLVVA
Il1r2 1 M--DLVYVNSDFVCSATASSDKSS----HDKGQKTFNKDH--HRWTKVLLIALTVCLLFA
Il1r3a 1 M--GSVYENSVFAFSTG----------HFSNYRESKREVNTAKWTKVLLIVFAVSLVFA
Il1r3b 1 M--GSVYENSVFAFSTGNSDFNDSQYKGHFSNYRESKREVNTAKWTKVLLIVFAVSLVFA
Il1rL 1 MSEDVVYSEVVFVKKKQADKDDED-----DPDQCDLQKRRSCCDGVRLVTTALCVIFTTG
Il1r5 1 M--DSVYENSRIVLSTVASNENSRN-EGNHSYEQESERREVNTAKWNKLLLTVFAVSLVFA
Il1r1 55 LGAVCTLAVFLAHAK-HYNVSVSD----------QEHNVTDYM
Il1r2 53 LGAVCTLAVFMARTETHGNVSVSD----------QEHNATDYK
Il1r3a 48 LGGLCAVRIQYVSVSAQLSAQETNGTIMSRQLDKLTANFTTVRDHLHINPLLMKELTANY
Il1r3b 59 LGGLCAVRIQYVSVSAQLSAQETN----------GTMLMKELTANY
Il1rL 56 LIALSFLCYMQATSNHNFQKKLQD----------LQEVHDALQENFTAFS
Il1r5 58 LGGLCVVSILYVRALTQQSAQTNTKIMKRQLEELTAN-CSRVKDDLYIKDSMMKDLTANY
Il1r1 87 EQLDVLRIQHQETLRKLNRLN------QSSGCALCAVRWTHSGGKCYYFSTVK--MNWTQ
Il1r2 86 EQLDVLHIQHQEMLQKLNRLN------ESSGCALCAVHWTHSGGKCYYFSTVK--MNWTQ
Il1r3a 108 SRVKEQLSISEETVRKLSRFN------QSSGCAICAIHWTHSGEKCYYFSTVK--MNWTQ
Il1r3b 95 SRVKEQLSISEETVRRLSRFN------QSSGCAICAIHWTHSGEKCYYFSTVK--MNWTQ
Il1rL 96 AVLEHIYNREQNLSRALNN----------SAQCPEDWQYHAGKCYYFSSNTNTLDWFK
Il1r5 117 SRVKDDIHIKDSMVKELTANNSRIKEQQSFYKAFRASNMTAFQGKLYFFSSDK--LTWSS
Il1r1 139 SRDHCVTKGGHLVIITSKAEQDFLTSNVKETH---WIGLNDLDTEGHWIWVDNQPLSQT-
Il1r2 138 SRDHCVTKGGHLVIITSKAEQDFLASKISVTH---WIGLNDMHTEGRWVWVDNQPLNKS-
Il1r3a 160 SRDHCVTKGGHLMIITSQAEQEFLTSNVKETH---WIGLNDLDTEGRWLWVDNQPLSQT-
Il1r3b 147 SRDHCVTKGGHLVIITSQAEQEFLTSNVKETH---WIGLNDLDTEGRWLWVDNQPLSQT-
Il1rL 144 SRDACISDGGHLVIINNRDEQEFLMSKTNKYKGSFWIGLTDKSTEGQWLWVDNTKLSTD-
Il1r5 175 SRAFCVSRGTDLVTITSRSEQAFLQSKMNEWT---WIGLSDLETEGRWVWVNNQTLNDLG
Il1r1 195 VEFWIKRENGVREPDNWT---KQHVDGEDCASLGHPGGETDFWTDAYCFQKKRFICEAAA
Il1r2 194 VEFWMKRVNGNNEPDNWT---KNHPGGEDCACLGHSLGATEFWNDDLCTATKRFVCEAAA
Il1r3a 216 EEFWMKRENGVSEPDNWT---KQHVDGEDCASLGHPDGETDFWTDAYCFEEKRFVCEAAA
Il1r3b 203 EEFWMKRENGVSEPDNWT---KQHVDGEDCASLGHPDGETDFWTDAYCFEEKRFVCEAAA
Il1rL 203 IRYWNGQ-----EPDNWKGYRNEYTEGEDCARIEQNNWNINSWFDAFCTIAFRRICETRS
Il1r5 232 VEFWYKRQSGKSEPDNWT---KDDPSGPHCAIVKYALNYLKSWFDVSCEHTYKFICEKKI
Il1r1 252 AV-STP
Il1r2 251 AINSTP
Il1r3a 273 AV-STP
Il1r3b 260 AV-STP
Il1rL 258 SR-STP
Il1r5 289 ---STP

B.



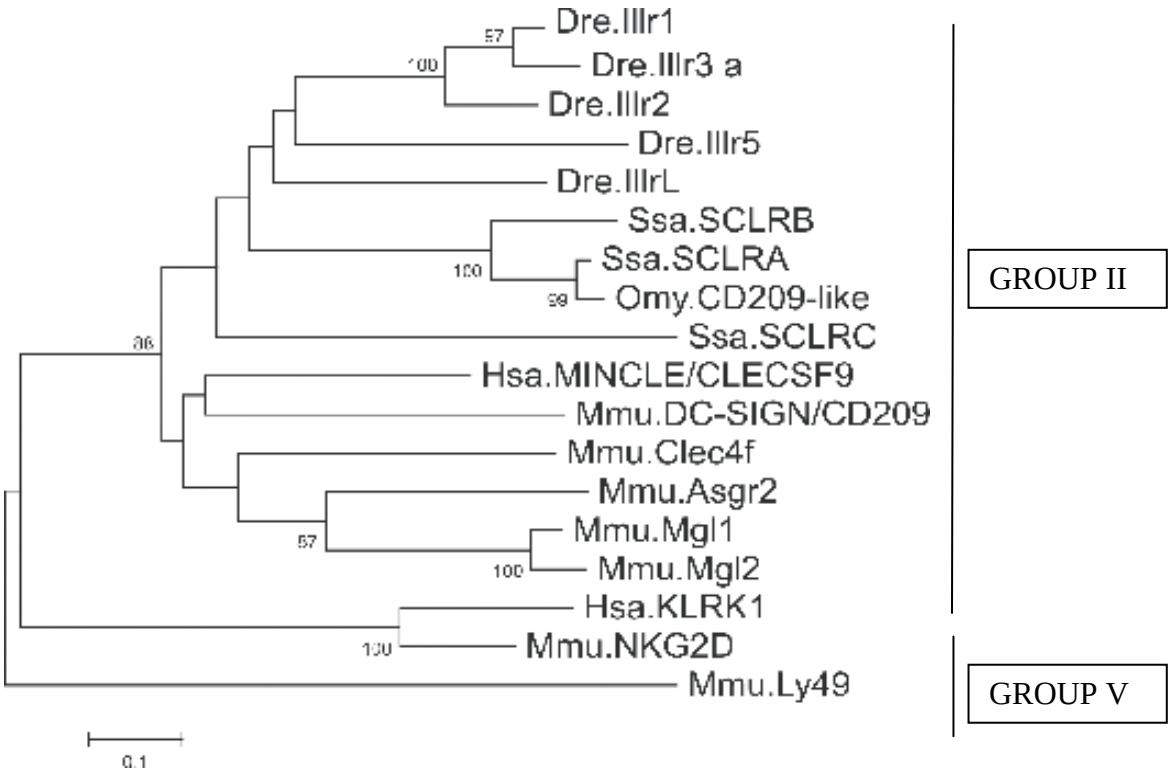


Figure 2. Phylogenetic analysis of zebrafish ILLRs to other C-type lectin receptors. CTLDs from zebrafish ILLRs were compared with CTLDs from other Group II or V C-type lectin receptors. Protein symbols are preceded by species symbols (*Dre: Danio rerio, Ssa: Salmo salar, Omy: Oncorhynchus, Mmu: Mus Musculus, Has: Homo sapiens*). Analyzed C-type lectin receptors include SCLRA, SCLRB, SCLRC, MINCLE/CLECSF9, DC-SIGN/CD209, CD209-like, C-type lectin domain family 4 member f (Clec4f), asialoglycoprotein receptor 2 (Asgr2), macrophage galactose N-acetyl-galactosamine specific lectin-1 (Mgl1), Mgl2, killer cell lectin-like receptor subfamily K member 1(KLRK1), NKG2D, and Ly49. Bootstrap values less than 80% are not shown.

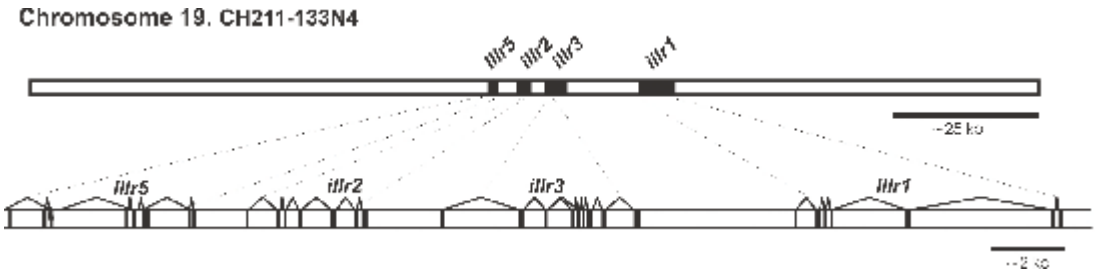


Figure 3. Genomic organization of *illr5* in zebrafish chromosome 19. The *illr5* gene is present in the ILLR gene cluster along with with *illr1*, *illr2*, and *illr3* encoded by BAC Ch211-133N4. The upper figure indicates the approximate position of ILLR in BAC211-133N4 and the lower figure shows their exon organization.

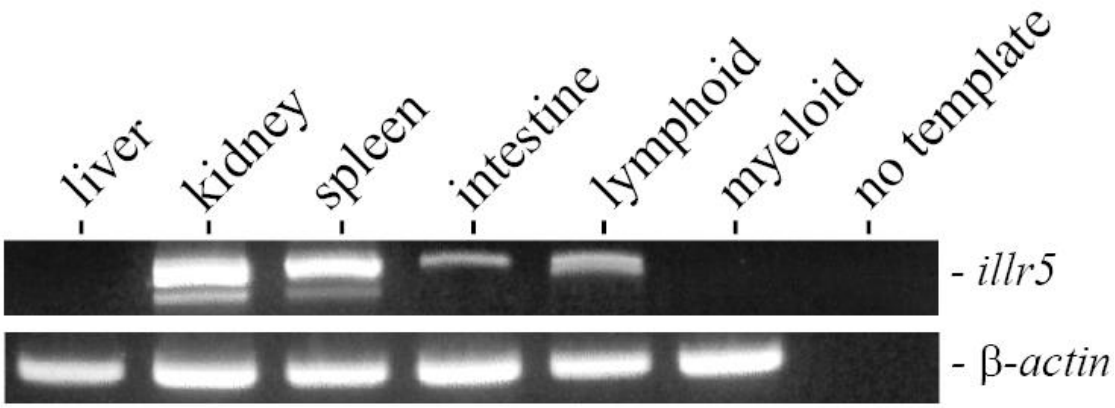


Figure 4. *illr5* is differentially expressed in adult zebrafish tissues and leukocyte lineages. *Illr5* GSPs were designed to amplify its cDNA sequence by RT-PCR on different tissues and FACS-sorted cellular lineages. Analysis of β-actin is included as a positive control.

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CHAPTER 6. DISSERTATION SUMMARY AND FUTURE DIRECTIONS

ILLRs in zebrafish were first characterized by Panagos et al. in 2006 (1). Identification of NK cells in bony fish through reverse genetic tools began more than a decade ago. As discussed above in the Introduction, NK cell receptors contain two classified families: Ig-domain receptors and C-type lectin-like receptors. NITRs representing Ig-domain receptors have been identified in at least 13 species of bony fish, and are possiblely present in all bony fish (2). They may be a good clue to explore the existence of NK cells in bony fish. On the other hand, ILLRs have Group 2 CTLDs plus intracellular motifs similar to ITIM, suggesting that they may shed a light on identification of NK cells through the lectin-like receptors. ILLRs are believed to play an important role in teleost innate immunity. Therefore, the work described in this dissertation focused on investigating the morphologic expression pattern and function of ILLRs in zebrafish.

The aims of the experiments described in Chapter 2 were to demonstrate the cellular expression patterns for the ILLR genes. To study this question, two cutting-edge techniques available in zebrafish research were utilized including RISH and fluorescently labeled transgenic fish lines. In the RISH experiments, 4 different protocols were used in either paraffin-embedded or frozen sections in order to identify ILLRs positive cells. Results showed either non-specific reactions or no signal in the spleen, kidney, and intestine. Experiments using GeneDetect Kits demonstrated the good RNA quality on frozen section when using the poly T probes. However, we could not detect any ILLR-specific cells with this protocol. Experiments that were performed in our collaborator's lab still failed to identify *illrL* positive cells even though the positive control probe TCRα showed a specific reaction.

These observations led us to suggest that ILLR genes could be expressed at low levels. Next, we built ILLRs transgenic fish lines with *I-SceI* meganuclease and Tol2 transposase system. Analysis of cells from F1 transgenic zebrafish (*illr1*^{10k}*:EGFP*) indicated an extremely low percentage of GFP positive cells in spleen/kidney based on anti-GFP Ab staining. At the same time we also saw a high GFP background in splenocytes and kidney cells, which reduced the possibility of successfully sorting ILLRs positive cell population. Taken together, these results suggest that ILLR genes are expressed at extremely low levels.

The series of experiments in Chapter 3 were designed to explore different ways to boost ILLRs gene expression. Previous studies from other laboratories have developed tools using zebrafish as a model to study infectious diseases (3, 4). We postulated that if ILLRs play similar roles as their mammalian counterparts in pathogen recognition and cellular adhesion, then their expression may increase upon immune challenging. With this in mind, bacterial infection with *S. iniae* and virual infection with SHRV were used and ILLR gene expression was analyzed in tissue of infected zebrafish by real-time PCR at several different time points post infection. We saw that *illrL* transcripts increased approximately 60-fold after *S. iniae* infection compared to the uninfected group starting at 8 hpi. There was still a marked increase in *illrL* at 48 hpi, and then *illrL* expression deceased to baseline levels at 72 hpi. Infection with SHRV led to increased *illrL* expression (about 35-fold increase at 12 hpi). We did not observe consistent changes for *illr1* and *illr3* in these studies. These results indicate two points: 1) *S. iniae* infection boosts *illrL* expression which may help us further

characterize its cellular pattern and function; 2) *illrL* could play different roles from *illr1* and *illr3* since they responded to immune challenge differently.

Studies described in Chapter 4 investigated of whether using the *S. iniae* infection model on the founder generation of transgenic zebrafish (*illrL*^{2k}*:mCherry*) would increase mCherry expression. A pilot study was carried out first to define if spleen cells from one single fish would be enough for analysis by flow cytometry. The answer to that question was yes, but the results also suggested a possibility that mixed spleen cells from 3 or more fish could increase the mCherry background. Transgenic zebrafish were infected with *S. iniae* and dissected at 12 hpi. Single cell resuspensions from individual fish spleen were subjected to analysis by flow cytometry. Results from these experiments demonstrated a trend of increased mCherry expression, but not at significant level (p = 0.07). This small population of mCherry positive cells will not be enough to be purified by cell sorting.

In Chapter 5 we reported studies that identified an additional ILLR gene on zebrafish chromosome 19. *Illr5* locates in the same BAC (CH211-133N4) as *illr1*, *illr2*, and *illr3*. The *illr5* ORF sequence contains 867 bp. The gene is composed of 289 amino acid residues, including a 6-residue signal peptide ITIM-like motif and a single CTLD of approximately 136 residues. Phylogenetic tree examination of ILLR CTLDs reveals that Illr5 belongs to the zebrafish ILLR family. RT-PCR analysis demonstrated the expression of *illr5* mRNA in the lymphoid hematopoietic lineage. Further study will be necessary to characterize Illr5 immune function within ILLRs gene family.

Overall, this thesis encompassed work designed to develop tools for identifying and characterizing ILLR expressing cells. An immune challenge with *S. iniae* successfully boosted *illrL* gene expression which may facilitate the identification and characterization of IllrL positive cells from zebrafish spleen tissue. Although these results did not allow us to characterize IllrL expression in transgenic zebrafish (*illrL*^{2k}*:mCherry*), future studies will focus on the development of other tools to facilitate IllrL identification with this infectious model. This may include mAb development. Previous work using a Nitr9 reactive mAb showed that this mAb worked well *in vitro* yet could not be used to purify Nitr9 positive cells *in vivo* by FACS (personal communication with Dr. Radhika Shah). Therefore, a better approach may be to find the ligand recognized by IllrL, which will help to answer a series of questions associated with IllrL function. This research has provided a tool to study IllrL function in zebrafish, which will pave the way to understand the significance of ILLRs and possibly provide insight into the host-pathogen interaction associated with the innate immune response in zebrafish. Furthermore, it might shed light on the evolution pathway of C-type lectin-like receptors in the innate immunity in vertebrates.

References

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APPENDIX

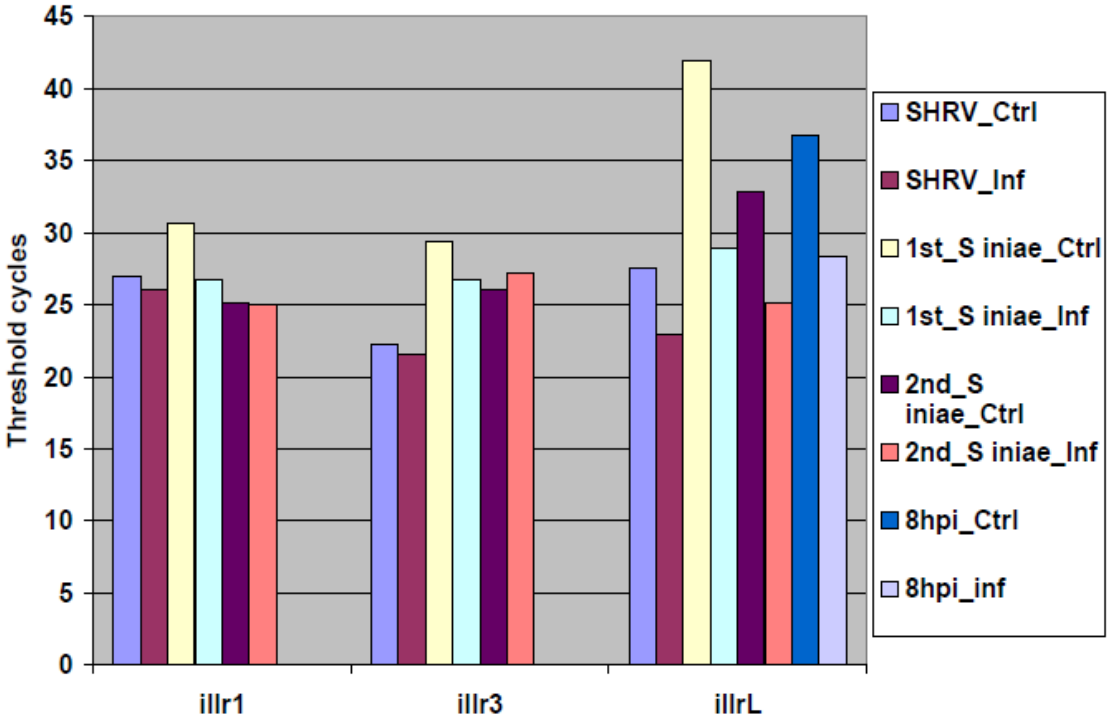

Figure 1. Threshold cycles for ILLRs at 8 hpi following either SHRV or *S. iniae* infection. Adult zebrfish were subjected to infection with SHRV and *S. iniae* infection. At 8 hpi, spleen tissues were dissected and RNA was isolated for real-time PCR analysis. Threshold cycle numbers were obtained from previous infectious studies in Chpater 3 and 4. SHRV_Ctrl vs SHRV_Inf compared the fold-induction after SHRV infection. 1^{st}_S iniae_Ctrl vs 1^{st}_S iniae_Inf compared the fold-induction during the first trial experiment in *S. iniae* infection study. 2^{nd}_S iniae_Ctrl vs 2^{nd}_S iniae_Inf compared the fold changes induced by the *S. iniae* infection in second trail experiment. 8hpi_Ctrl vs 8hpi_Inf compared the fold changes in *illrL* only induced by *S iniae* infection which was performed in our own lab.

Figure 2. *illr2* and *illr5* full cDNA sequences

>*illr2*_cDNA (JY1202)
ACTTGGAGACTCAGAAGTAAGATATCCGTCAGGCTAATAAAATGGATCTGGTTTATGTCAATTCTG
ATTTTGTATGTTCAGCAACTGCTTCAAGTGACAAAAGCTCACATGACAAAGGTCAAAAAACCTTCA
ATAAAGATCATCATCGATGGACTAAAGTTCTTTTGATTGCTCTCACTGTGTGTCTTTTATTTGCTCT
CGGAGCTGTCTGCACCCTGGCCGTGTTTATGGCACGCACAGAAACACATTCAGATTTCAAATGTGT
CTGTGTCTGATCAAGAACACAATGCCACAGGTCAGTAAAAAAAGCAACCACGAAGTGATTTCATT
TCTGCTTTTGTGCACAGAATAAAACTTGTGAAAAACTTGATCACTTACTATATATGCATTCATTCAT
TCATTTTCTTGTCGACTTAATCCCTTTATTAATCCGGGGTCGCCACAGCGGAATGAACTGCCAACTT
ATCCAGCAGGTTTTTACGCCCTTCCAGCCGCAAGCCATCTGTGGGAAATACTATATATGCATTTCT
ATTTATTTATTTTTTTTGCATGCAGATTACAAGGAACAGCTTGATGTGCTGCACATTCAGCATCAGG
AGATGCTTCAAAAGCTGAACAGATTAAACGAAAGCAGCGGCTGTGCACTCTGTGCAGTTCACTGG
ACTCATTCTGGAGGAAAGTGTTACTACTTCTCTACAGTCAAGATGAACTGGACACAGAGTCGAGA
TCACTGTGTGACCAAAGGAGGACATTTGGTGATCATAACCAGCAAAGCAGAGCAGGATTTCCTTG
CCTCTAAAATTTCAGTAACCCACTGGATTGGTCTTAATGATATGCACACAGAGGGACGCTGGGTTT
GGGTGGACAACCAGCCACTTAATAAATCAGTAGAATTCTGGATGAAACGAGTGAATGGGAACAAT
GAACCTGATAACTGGACTAAAAATCATCCCGGTGGAGAGGACTGCGCTTGCCTGGGTCACTCTTTG
GGAGCAACTGAATTCTGGAACGATGATCTGTGTACTGCAACGAAAAGATTTGTGTGTGAAGCCGC
TGCGGCCATTAATTAA

>*illr5*_C28 (JY1151-T7)
AGGCTCATGAAATGGACTCAGTCTATGAAAATTCTAGAATCGTCCTTTCAACGGTTGCTTCAAATG
AAAACTCTCGGAATGAAGGAAACCACAGTTATGAGCAAGAAAGTGAGAGGGAAGTCAATACCGC
AAAGTGGAATAAACTTCTGCTGACTGTTTTTGCTGTGTCTCTGGTTTTTGCTCTTGGAGGTCTCTGT
GTTGTAAGCATACTGTATGTCAGGGCTTTAACGCAACAGTCTGCCCAAACTAACACCAAAATCATG
AAGAGACAGCTTGAGGAACTGACAGCAAACTGCAGCAGAGTTAAAGATGACCTCTACATTAAAG
ACTCCATGATGAAAGATCTGACAGCGAACTACAGCAGAGTTAAAGATGATCTCCACATTAAAGAC
TCCATGGTGAAAGAGCTGACAGCTAACAACAGCAGAATTAAAGAACAACAGTCCTTTTATAAAGC
ATTTAGAGCATCAAACATGACAGCTTTTCAAGGAAAGCTGTATTTCTTCAGCTCTGATAAACTAAC
TTGGTCAAGCAGTCGAGCTTTCTGTGTTTCAAGAGGAACCGATCTGGTCACCATAACCAGCCGATC
TGAACAGGCATTTCTGCAATCTAAAATGAACGAATGGACATGGATTGGACTCAGTGATCTGGAGA
CTGAAGGACGTTGGGTTTGGGTGAACAATCAGACTCTTAATGACACTGGAGTAGAGTTTTGGTAC
AAGAGGCAATCTGGGAAAAGTGAACCTGACAACTGGACAAAGGATGATCCCTCTGGAGAACACT
GTGCTATTGTGAAATATGCTCTCAACTATTTAAAAAGCTGGTTTGATGTTTCTTGCGAGCATACAT
ACAAGTTCATCTGCGAAAAGAAAATTTAAGTCAGTCAAAATTTAATTCCATCAATTGGAGCTAATT
AATAAATTCTATTCTACAGAGAAAACTAAACTCCTCTTCTGGATTTGCTCACTCAATGTCAGGCTG
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