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Examination of the signalling properties and ligand-binding potential of stimulatory leukocyte immune-type receptors (IpLITRs) in the channel catfish (*Ictalurus punctatus*)

by

Jacqueline Mewes-Arès

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Dr. James Stafford, Biological Sciences

Dr. Andrew Keddie, Biological Sciences

Dr. Luis Schang, Biochemistry

ABSTRACT

Channel Catfish (*Ictalurus punctatus,* Rafinesque, 1818) leukocyte immune-type receptors (IpLITRs) are a family of proteins sharing structural and phylogenetic relationships with mammalian immune receptors. Based on their predicted signalling potential and ligand-binding properties, IpLITRs may be important in the control of immune cell effector responses in fish. The main objectives of this thesis were to determine how stimulatory IpLITRs activate cells and to develop assays for the screening of IpLITR ligands. Using cellular transfections, co-immunoprecipitation, and flow cytometry, I determined that stimulatory IpLITRs associate with specific adaptor molecules, which is required for their surface expression and signalling ability. These adaptors assemble with IpLITRs via their charged transmembrane regions and contain cytoplasmic tails encoding tyrosines that may initiate kinase pathways leading to immune cell activation. This study represents the first step towards elucidating how IpLITRs 'turn on' immune cells. Combined with the development of assays to identify IpLITR ligands, my work sets the stage for further investigations into the functional characterization of these receptors.

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ADCC:	antibody-dependent cell-mediated cytotoxicity
ASB:	antibody staining buffer
β2m:	beta 2 microglobulin
BCIP:	5-bromo-4-chloro-3-indolyl phosphate substrate
BSA:	bovine serum albumin
Btk:	Bruton's tyrosine kinase
CD3ζ-L:	CD3 zeta-like molecule
CHIR:	chicken immunoglobulin-like receptor
Clr:	C-type lectin related molecule
CMV:	cytomegalovirus
CYT:	cytoplasmic tail
DAP10:	DNAX-activating protein 10
DAP12:	DNAX-activating protein 12
DMEM:	Dulbecco's modified Eagle's medium
D-PBS:	Dulbecco's phosphate buffered saline
DrCD3ζ:	zebrafish CD3 zeta chain
DrDAP12:	zebrafish DAP12
DrFcRγ:	zebrafish Fc receptor gamma chain
DrFcRγ-L:	zebrafish Fc receptor gamma chain-like molecule
EDTA:	ethylenediaminetetraacetic acid
ELISA:	enzyme-linked immunosorbent assay
ER:	endoplasmatic reticulum
EST:	expressed sequence tag
Fab:	fragment antigen binding region
FACS:	fluorescence-activated cell sorting
FBS:	fetal bovine serum
Fc:	fragment crystallizable
FcαR:	Fc alpha receptor
FcδR:	Fc delta receptor
FcεR:	Fc epsilon receptor
FcγR:	Fc gamma receptor
FcμR:	Fc mu receptor
FcR:	Fc receptor
FcRγ:	Fc receptor common gamma chain
FcRγ-L:	Fc receptor gamma-like molecule
FCRL:	Fc-receptor-like molecules
FL:	fluorescence
GFP:	green fluorescent protein

H + L:	heavy and light chain
HA:	hemagglutinin
HIV:	human immunodeficiency virus
HLA:	human leukocyte antigen
HRP:	horse radish peroxidase
Ig:	immunoglobulin
IgSF:	immunoglobulin superfamily of receptors
IL2:	interleukin 2
IP:	immunoprecipitation
IpCD3ζ-L:	channel catfish CD3 zeta-like molecule
IpDAP12:	channel catfish DAP12
IpFcRγ:	channel catfish Fc receptor gamma chain
IpFcRγ-L:	channel catfish Fc receptor gamma chain-like molecule
IpLITR:	channel catfish leukocyte immune-type receptor
ITAM:	immune receptor tyrosine activation motif
ITIM:	immune receptor tyrosine inhibition motif
KIR:	killer immunoglobulin-like receptor
LB:	Luria-Bertani
Lck:	leukocyte-specific tyrosine kinase
LILR:	leukocyte immunoglobulin-like receptor
LRC:	leukocyte receptor complex
mAb:	monoclonal antibody
MHC I:	major histocompatibility complex class I
NBT:	nitro blue tetrazolium
NFAT:	nuclear factor of activated T cells
NITR:	novel immune-type receptor
NK:	natural killer
NKR-P1:	natural killer cell receptor P1
Ocil:	osteoclast inhibitory lectin
pAb:	polyclonal antibody
PBS:	phosphate buffered saline
PCR:	polymerase chain reaction
PE:	phycoerythrin
PH:	pleckstrin homology
PI3-K:	phosphoinositol-3-kinase
PIR:	paired immunoglobulin-like receptor
PKC:	protein kinase C
PLC:	phospholipase C
PPT:	preprotrypsin
PtdIns(3,4,5)P3:	phosphatidylinositol-3,4,5-trisphosphate

PtdIns(4,5)P2:	phosphatidylinositol-4,5-bisphosphate
Ptk:	Src family phosphotyrosine kinase
RT-PCR:	reverse transcriptase polymerase chain reaction
SDS-PAG:	sodium dodecyl sulfate polyacrylamide gel
SDS-PAGE:	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SH2:	Src homology 2 domain
SHIP:	Src-homology-2-domain-containing inositol-5 phosphatase
SHP:	Src-homology-2-domain-containing tyrosine phosphatase
SOS:	son of sevenless homologue
SP:	signal peptide
Syk:	spleen tyrosine kinase
TAE:	Tris base, acetic acid and EDTA buffer
TBS:	Tris-buffered saline
TCR:	T cell receptor
TM:	transmembrane domain
TREM:	triggering receptor expressed on myeloid cells
TTBS:	Tris-buffered saline supplemented with Tween 20
TTBS-SKIM:	Tris-buffered saline supplemented with Tween 20 and skim milk powder
XFR:	frog Fc-like receptors
ZfLITR:	zebrafish leukocyte immune-type receptor

CHAPTER 1
INTRODUCTION

1.1 Introduction

The immune system of vertebrates is comprised of an innate and an adaptive arm. While the innate immune system provides an immediate defence against invading pathogens and the detection and destruction of tumours, activation of cells of the adaptive immune system is significantly delayed [1]. The adaptive immune system depends on the activation and proliferation of immune cell subsets that provide a vigorous and long lasting response, culminating in what is known as immunological memory. The concerted action of the innate and adaptive immune systems provide robust immunity to all vertebrates [2]. Cells of the innate immune system include dendritic cells, Natural Killer (NK) cells and monocytes, such as macrophages, whereas the cells of adaptive immunity are B cells and T cells. Immune cells receive activating and inhibitory signals. For both innate and adaptive immunity it is the balance between immune cell activation and suppression that ultimately determines the physiological outcome, be it immune tolerance or immunological defence [3]. The mechanisms behind activation and/or inhibition of an immunological response at the cellular level and their timing are critical. Failure within these mechanisms can result in immunosuppression [4] or auto-immunity [5], and understanding how these immunological responses are initiated is an active area of research.

Immunoregulatory receptors are cell surface proteins that mediate cellular processes in all immune cell types. Studies of these molecules have given us an appreciation for the central role of these receptors in mediating critical immune cell responses [6-8] and have also helped gain important insights into the conservation of immune cell signalling networks among vertebrates [9-11]. This knowledge of vertebrate immune receptor signalling is therefore essential in

bridging the gaps in our understanding of the evolution of the immune system and also in advancing studies in animal as well as human health.

Teleost or bony fish are important immunological model organisms and aquatic animals with significant industrial relevance. Fish such as salmon and catfish provide an important component in human nutrition and are farmed in many countries across the world. However, very little is known about the regulation of fish immune cell functions. Contributions to the understanding of fish immunoregulatory receptors will not only advance aquaculture in allowing for breeding of disease resistant fish but can also help advance the field of aquatic toxicology. Here, aquatic organisms can serve as sentinels in studying the effects of toxic components and associated immune responses in the aquatic environment. The channel catfish (*Ictalurus punctatus*, Rafinesque 1818) is a particularly attractive model in the field of teleost immunology, since its immune system, to date, is the most characterized of any teleost or ectothermic vertebrate, for that matter. The functional characterization of channel catfish immunoregulatory receptors has therefore great potential in benefiting the field of comparative immunology, physiology and also the applied fields of aquaculture and environmental toxicology.

1.2 Objectives of the thesis

The discovery of a large family of receptors termed leukocyte immune-type receptors (IpLITRs) from immune cells of the channel catfish has prompted many questions regarding their function. The cloned IpLITRs have been subjected to phylogenetic analyses and homology modelling, but their functional relevance in regulating immune effector functions is unknown. The main objective of my thesis was to develop the tools that aid in the functional characterization of a subset of IpLITRs with regards to their signalling potential and their ligand binding properties. The specific aims of my thesis research were:

1) To determine which ITAM-containing signalling adaptor molecule(s) associate with a putative stimulatory IpLITR-type.
2) To generate soluble IpLITR-Fc fusion constructs as staining agents and to test for binding of soluble IpLITR-Fc fusion constructs to target cells.
3) To generate IpLITR-CD3ζ constructs for expression in BWZ reporter cells.
4) To clone channel catfish MHC I α chain and β2 microglobulin (β2m) from a B cell line and express them as recombinant proteins in the human HEK 293T cell line.

1.3 Outline of the thesis

In the second chapter of my thesis, I review the role of immune receptors in mediating critical cellular responses. Specific emphasis is placed on inhibitory and stimulatory receptors, receptors that bind MHC I and receptors that bind immunoglobulins (Ig). In the same chapter, I also describe the channel catfish as a unique vertebrate model for comparative immunological studies and review the current literature on IpLITRs (e.g. what is currently known of their inhibitory signalling potential and their putative binding partners). Chapter 3 contains the description of all the materials and methods used throughout this thesis. In Chapter 4, I describe the results of IpLITR-adaptor association studies. Specifically, in this chapter I demonstrate that a putative stimulatory IpLITR-type (i.e. IpLITR 2.6b) associates with teleost FcRγ and CD3ζ homologues, but the surface expression of IpLITR 2.6b is increased only in co-transfections with FcRγ homologues. In Chapter 5, I describe the results of strategies undertaken to identify IpLITR ligands. I also report the generation of soluble IpLITR-Fc fusion proteins and the results from several staining attempts of target cells. The generation of IpLITR-CD3ζ fusion constructs for expression in BWZ reporter cells is also described in Chapter 5 along with the cloning and expression of

channel catfish MHC I in HEK 293T cells in an attempt to generate a target cell that allows testing for this putative IpLITR ligand. The final chapter is a general discussion on the insights that my studies have provided towards the functional characterization of IpLITRs and I also discuss the implications for future studies to further characterize this family of teleost immune receptors.

CHAPTER 2
LITERATURE REVIEW

2.1 Introduction

Immune receptors act as mediators of cellular effector responses by the initiation and inhibition of a wide array of intracellular signalling cascades. These surface proteins sense a variety of signals from their environment, such as inflammatory cytokines, chemokines and other proteins, including antibodies, produced by the immune system. [12-14]. Immune receptors are also involved in the detection and elimination of extracellular pathogens and surveillance of host cells for signs of damage. Consequently, they play an important role in self/non-self recognition [15, 16], the identification of stressed cells [17] and in the detection of viral components expressed on or within infected cells [18-21]. The vast array of immune receptors responsible for these events varies greatly among the different immune cell types.

This chapter reviews the key features of innate immune receptors with a particular emphasis on their inhibitory and stimulatory signalling capabilities. I then describe innate immune receptors within vertebrate taxa focussing on two important groups of ligands that they recognize: MHC I complexes and immunoglobulins (or antibodies). Next, the key features of the channel catfish as a unique immunological model are highlighted followed by an introduction of the channel catfish LITRs. The current knowledge of IpLITR signalling potential and their putative binding partners/ligands will also be described.

2.2 Innate immune receptors

The signalling pathways within innate immune cells are diverse and display an enormous array of complexity [3]. Regardless of this complexity, the initiation of cellular signalling events often requires specific immune receptor-ligand

interactions that set off the cascade which will ultimately determine the fate of cellular responses. Immune cells express diverse subsets of type I transmembrane proteins on their surfaces. These proteins consist of extracellular domains that bind soluble ligands or binding partners expressed on the surface of target cells. The surface expression of these receptors is facilitated by a signal peptide and sometimes requires the association with another auxiliary molecule or adaptor. Examples of innate immune receptor-types expressed by mammalian immune cells, such as NK cells, include killer Ig-like receptors (KIRs), leukocyte immunoglobulin-like receptors (LILRs) as well as Fc receptors (FcRs). Innate immune receptor families are either members of the immunoglobulin-superfamily (IgSF), which contain extracellular immunoglobulin (Ig) domains or C-type lectin receptors. Interestingly, many of these immune receptor families are arranged in gene clusters, best studied in humans [22-24]. The existence of immune receptor gene clusters has also been shown in other vertebrates such as mice, dogs, opossums, chickens, [11, 25] amphibians [26] and fish [27, 28].

The co-expression of stimulatory and inhibitory immunoregulatory receptor-types on NK cells, T cells, monocytes, and dendritic cells has been described for the mammalian immune receptors of the KIR [29, 30], LILR [31], paired Ig-like receptor (PIR) [32], and the FcR gene families [33]. In birds, amphibians and fish, several structurally related multigene immunoregulatory receptor families with putative dual signalling capabilities have also been described [25, 26, 34, 35]. This suggests a conserved trend within the function of these receptors. The co-expression of inhibitory and stimulatory receptor-types of the same family is important in setting an activation threshold in order to achieve controlled cellular responses [36].

2.2.1 Stimulatory receptors and their signalling adaptors

The activation of an immune cell is generally initiated through ligand binding by stimulatory receptor-types [37]. Some stimulatory immune receptors contain

intrinsic cytoplasmic signalling motifs, whereas others lack signalling motifs but instead recruit signalling adaptors as mediators of cellular activation [8, 37]. Several mammalian stimulatory innate immune receptors that associate with signalling adaptors are in the FcR γ family (FcγR), KIRs, the Ly49 receptor family or the NK cell receptor family: FcγRIIIa/b [38, 39], KIR2DS [40], Ly49D, Ly49H [41] and NKG2D [42].

Signalling adaptors often consist of a short extracellular region, a transmembrane segment (TM) and a cytoplasmic tail containing one or more immune receptor tyrosine-based activation motif (ITAM). The basic consensus sequence of which is YxxL/I(x_{6-8})YxxL/I (where x denotes any amino acid). Intracellular signalling is mediated through these adaptors via enzyme cascades including phospholipase C (PLC) and phosphoinositol-3-kinase (PI3-K) [43, 44]. The signalling adaptors CD3ζ, FcR γ chain (FcRγ chain) and DAP12 act through such mechanisms. Alternatively, the YxxM motif in the cytoplasmic tail of the signalling adaptor DAP10 acts as a docking station for PI3-K. Recruitment of PI3-K then sets off a kinase cascade resulting in activation of the immune cell [45]. Stimulatory immune receptors that recruit signalling adaptors contain a positively charged residue within their TM segment (a lysine or an arginine) and their associating signalling adaptors contain an aspartic acid residue, conferring a negative charge to their TM region [46]. These opposite charges appear to be critical to allow the non-covalent association between receptor and adaptor molecules. The aspartic acid residue in the TM of the adaptor molecule and the arginine or lysine within the TM of the immune receptors are thought to form salt bridges. These bridges aid in stabilizing immune receptor-adaptor complexes in the plasma membrane. A stable receptor complex can only form on the cell surface when the receptor molecule is paired with the physiologically relevant adaptor. Despite similarities between adaptor TM regions, the specificity in adaptor-receptor paring indicates that the conserved aspartic acid residue in the TM domains of adaptors do not permit promiscuous pairing. Complex interactions may dictate the orientation of the contact sites within the TM

segments of receptor and adaptor molecules. There is also different spacing between positively charged residues within the TM domains of adaptors and receptors which may contribute to specific conformation for pairing with the corresponding receptor [47].

Upon ligand binding by the stimulatory immune receptor, tyrosine residues within the ITAM of the associated adaptor are phosphorylated by a membrane associated src homology protein tyrosine kinase. This phosphorylation recruits intracellular kinases such as Zap-70 or Syk, which then signal further in initiating cascades to activate PLC or PI3-K and results in the generation of phosphoinositol-3 molecules. Furthermore, Ras, Map kinase or protein kinase C (PKC) are activated in these signalling cascades [8, 48]. Signalling adaptors that contain an YxxM motif, recruit PI3-K directly to the adaptor molecule and initiate a signalling cascade through the immediate formation of phosphoinositol-3 molecules [49].

The signalling adaptor molecule CD3ζ was originally identified as part of the T cell receptor (TCR) complex of CD3 [50]. The CD3ζ adaptor forms a homodimer via a disulfide bond and is necessary for the receptor complex to reach the cell surface. Without the CD3ζ subunits, the pentameric αβγδε complex is assembled in the endoplasmatic reticulum and then transported to the lysosomes. Therefore, the CD3ζ adaptor subunit provides signalling potential to the TCR via its three ITAM motifs, but it also prevents TCR degradation [51]. The CD3ζ adaptor is also a player in immune signalling in NK cells by acting as a signalling adaptor for the NK FcR called CD16 [39, 44].

Like CD3ζ, the FcRγ chain is also a disulfide linked dimer that acts as a signalling adaptor for the high affinity IgE receptor FcεRI. This well studied innate immune receptor consists of an α, a β and two γ subunits. Analyses of the gene structures of the CD3ζ chain and the FcRγ chain reveal that these genes have evolved from a common ancestor by gene duplication [39]. In humans, both genes are localized on chromosome 1, displaying similar genomic organization [39].

The FcRγ chain is also expressed by various immune cells such as NK cells, dendritic cells, B cells, T cells, mast cells, monocytes, macrophages and even osteoclasts [44]. In mammalian immune cells, the FcRγ chain associates with an array of functionally distinct immune receptors including FcγRI, FcγRIII, FcεRI, mouse PIR-A, human LILR, KIR2DL4, NKp46 and dendritic cell activating receptor [44, 52]. Signalling upon ligand binding induces receptor clustering and adaptor phosphorylation. This can lead to different effector functions depending on the type of immune cell. In NK cells, functions like cytotoxicity and release of cytokines [53] can be triggered. Internalization of receptor antigen complexes is an important effector function in monocytes [54]. This shows the importance of FcRγ chain in immunological elimination of target cells through NK-mediated cytotoxicity killing and monocyte/macrophage phagocytosis (Fig. 2.1).

DAP12 is another ITAM bearing signalling adaptor that forms disulfide linked dimers. Like the FcRγ chain, DAP12 is expressed by many cells of the immune system [45]. The list of mammalian immune receptors that associate with DAP12 as their signalling subunit includes TREM2, Ly49, KIR-S, CD94/NKG2C, NKp44 [44]. DAP12 signalling via Syk plays a major role in mediating NK cell cytotoxicity. Furthermore, DAP12 seems to be involved in cytokine release and calcium mobilization in immune cells [44].

DAP10 is a transmembrane signalling adaptor that mediates signalling events through its intracellular YxxM motif. This motif is identical to the signalling motif contained within the cytoplasmic tail of CD28. CD28 is a co-stimulator of T cells. The YxxM motif of CD28 activates PI3–K signalling [47]. Like the previous described signalling adaptors, DAP10 forms a disulfide linked dimer. The protein structure is very similar between DAP10 and DAP12. However, the YxxM motif in human DAP10 has potential to bind PI3-K, Grb2 and Shc, kinases involved in cellular activation cascades [45] (Fig. 2.2). DAP10 is expressed by NK cells, monocytes, T cells and B cells. The best studied example of a receptor with which DAP10 associates is NKG2D. DAP10 mediated NKG2D

receptor signalling has been suggested to play a role in the activation of NK and T cells [47].

2.2.2 Inhibitory receptors

In mammals, inhibitory immune receptors are vital for the attenuation of immune responses and preventing inappropriate cell-mediated tissue damage during inflammatory responses. Structurally, many of these inhibitory receptor-types belong to the IgSF. Within their cytoplasmic tails, they contain intracellular signalling motifs termed immune receptor tyrosine-based inhibition motifs (ITIM) with the consensus amino acid sequence of (S/I/V/L)xYxx(I/V) (where x denotes any amino acid) [37]. Following ligand binding to the inhibitory receptor, Src family phosphotyrosine kinase (Ptk)-dependent phosphorylation of the tyrosine residues within the ITIM allows binding of phosphatases to the receptor [55, 56]. Phosphorylation of members of the KIR family, for instance, depends on leukocyte-specific tyrosine kinase (Lck) activity, but not spleen tyrosine kinase (Syk) [57]. The Src-homology-2-domain-containing (SH2) tyrosine phosphatases such as SHP-1, SHP-2 or SH2-domain-containing inositol-5 phosphatase (SHIP) are recruited to the phosphorylated ITIM. SHP-1 dephosphorylates substrate proteins such as a phosphorylated stimulatory signalling adaptor and other Src kinase substrates [57]. The phosphoinositide hydrolyzing action of SHIP, on the other hand, results in the inactivation of pleckstrin homology containing proteins such as Bruton's tyrosine kinase (Btk) and PLCγ [58, 59]. As a consequence, calcium signalling is suppressed through reduced calcium influx from calcium stores [37, 59] (Fig. 2.3). These phosphatases can thereby attenuate, terminate or inhibit immune responses exhibited by various immune cells.

2.2.3 Stimulatory and inhibitory innate immune receptors that bind immunoglobulins

Receptors that bind immunoglobulins or antibodies can be found throughout vertebrates [11, 26, 60, 61]. The majority bind the Fc portion of circulating monomeric antibodies or antibodies bound in immune complexes. These receptors are therefore termed Fc receptors (FcRs). They mediate adaptor-dependent stimulatory signalling events within immune cells. The relevance of FcRs in innate immunity becomes most obvious when taking a close look on NK cell and macrophage functions. In these two key players of innate immunity, FcRs mediate a variety of effector responses. Recognition of the Fc antibody region is critical in identifying opsonised cells (cells with antibodies bound to their surface) or particles against which the adaptive arm of immunity has generated antibodies. For example, professional antigen presenting cells capture opsinized antigens via their FcRs, internalize and process these complexes and then display the antigens on their surface [11]. Antibodies bind bacterial or viral proteins on the surface of infected cells via their antigen binding region (Fab). Thereby, they label infected cells for destruction [62-64]. The destruction of infected cells is, in turn, mediated via FcRs on the surface of cytotoxic immune cells in a process referred to as antibody-dependent cell-mediated cytotoxicity (ADCC) [65].

The identification of FcR orthologues between different animal species poses a particular challenge in expanding the studies of FcRs across vertebrates. This is due to the vast differences between the Ig isotypes and subtypes present in the different species as they are identified to date. Mammals express isotypes IgA, IgD, IgE, IgG and IgM [66, 67], birds carry IgA, IgM and IgY [68], whereas *Xenopus leavis* isotypes are IgM, IgY and IgX [69, 70]. Bony fish express IgD [71-73] and IgM [74-76] as well as IgZ/IgT [77] isotypes. Receptors have been identified for some, but not for all of these antibody classes in these various animals [11, 26, 60].

In mammals, IgA antibodies are recognized by Fc α receptors (FcαR), IgG subclasses are recognized by various Fc γ receptors (FcγR), IgD is recognized by Fc δ receptors (FcδR) and IgE is recognized by Fc ε receptors (FcεR). In mice and humans, Fc receptor α/μ (Fcα/μR) recognizes IgA as well as IgM [78, 79]. This novel receptor is expressed in hematopoietic as well as non-hematopoietic tissues [80]. More recently, an IgM receptor (FcμR) has been identified in humans [81]. Within these Fc receptor groups, there are a range of receptor molecules with variable affinities for the different subclasses of a given antibody isotype [8]. Some FcRs bind antibodies with high affinity, others with medium to low affinity [82-85]. Crystal structures of several human FcRs have been resolved and revealed that the overall structure of these different receptors is closely related [85]. Low affinity receptors (e.g. FcγRIIA, FcγRIIIA) contain two extracellular Ig domains (D1 and D2), whereas the high affinity FcγRI contains an additional Ig domain (D3), which contributes to high-affinity binding [86].

In humans, the genes encoding classical FcRs recognizing IgG, IgE and IgA/IgM are localized in a gene cluster on chromosome 1q21.1-24 [79]. Within the diverse FcγR family in humans, there are receptor molecules with ITIMs, ITAMs as well as receptors without intracellular signalling motifs. Those receptors without signalling motifs often have the ability to recruit FcRγ chain or CD3ζ signalling adaptors via TM segment interactions [11].

A central and well studied role in antibody mediated killing in human NK cells has been attributed to FcγRIIIA. Human FcγRIIIA is an activatory immune receptor consisting of two extracellular Ig domains (D1, D2) and a single transmembrane segment. The receptor binds human IgG1 and IgG3 with variable affinity for subclasses. FcγRIIIA associates with CD3ζ in NK cells but with the FcRγ chain dimer in the cell membrane of monocytes and macrophages where it may have a role in phagocytosis. The crystal structure of FcγRIIIA co-crystallized with its binding partner IgG1 shows that the FcγRIIIA D2 domain contacts the Cγ2 and Cγ3 domains of the two heavy chains of IgG1. The crystal structure of

the FcγRIIIA IgG1 complex also revealed that the receptor binds its antibody ligand in its monomeric form in an asymmetrical manner that prevents other FcγRs to bind the same antibody molecule. This finding further supports the importance of immune complex binding for cellular activation following low affinity FcγR binding, since many binding events on multiple FcR molecules are required for immune cell activation, and only these immune complexes have multiple Fc fragments present that can be bound by the FcRs on the immune cell surface [8].

FcγRIIB, is an inhibitory Fc receptor and the best studied representative of ITIM containing FcRs [59]. FcγRIIB appears to be a key player in B cell survival. It also regulates antibody production, lymphokine release and cellular proliferation. In humans, this receptor is also expressed on a small NK cell subpopulation where it modulates FcRγ chain mediated functions [87]. NK cells that express FcγRIIB at high levels show weaker antibody dependent degranulation upon incubation with IgG-coated target cells in comparison to NK cells with low levels of FcγRIIB expression [87]. The overall role of this small NK cell subset in NK cell function, or whether there is a major role to these inhibitory subtypes of NK cells, is still unclear.

2.2.4 Stimulatory and inhibitory innate immune receptors that bind MHC I

Major histocompatibility class I (MHC I) molecules are highly polymorphic and are present in several haplotypes in humans [88-90]. These MHC I protein complexes display intracellular derived peptides on the surface of cells [91, 92]. Ubiquitinated naturally occurring cellular or pathogen derived proteins will be degraded by the cellular proteasome. The resulting peptides are transported into the endoplasmatic reticulum where the MHC I complex is assembled. This complex consists of a heavy chain (in this thesis referred to as α chain) containing

three Ig domains and a TM, a β2 microglobulin (β2m) and the intracellular derived peptide loaded onto the peptide binding groove of the α1 and α2 domains of the heavy chain. Within the adaptive arm of immunity, receptors such as the TCR 'scan' MHC I for the type of peptide present on the heavy chain of the complex [93]. In contrast, innate immune receptors scan for presence or absence of the MHC I complex itself on target cells [94, 95]. This may be a result of pathogen-host co-evolution applying selective pressure on pathogens to evade existing host defence strategies of antigen recognition through MHC I presentation to the TCR. As a consequence, some virally infected cells have a reduced display of viral peptides via MHC I due to down-regulated MHC I expression [96]. Hepatitis B virus, HIV-1, Karposi's sarcoma associated herpes virus and adenovirus are examples of viruses that down-regulate MHC I mediated antigen display on infected cells [96, 97]. However, the missing MHC I surface expression does not go unnoticed. NK cells express a great repertoire of inhibitory immune receptors that recognize MHC I. Missing self-recognition results in the activation of these NK cells and subsequent killing of the target cell. Among the innate immune receptors that bind MHC I in mammals are Ly49 and PIRs in mice, as well as human KIRs along with LILRs [94, 98, 99].

KIRs are a well studied receptor family that bind MHC I molecules and are expressed on NK cells as well as T cell subsets in humans as well as primates. The MHC I (HLA class I) ligands of these receptors are polymorphic, and so are the 16 members of the KIR gene family [30]. KIRs are members of the IgSF of receptors and their genes are located on human chromosome 19q13.4 [100-102]. Based on the number of extracellular Ig domains, two subfamilies of KIRs can be identified: KIR3D, containing three extracellular Ig domains and KIR2D, containing two extracellular Ig domains [103]. Within these subfamilies, there is diversity in their extracellular domains and throughout their TM and intracellular domains [30, 104]. There are ITIM bearing KIRs (e.g. KIR2DL and KIR3DL) in both subfamilies and stimulatory KIR types that lack an intrinsic signalling motif (KIR2DS and KIR3DS) but recruit signalling adaptor molecule DAP12 [105,

106]. Moreover, KIR2DL4 has characteristics of both inhibitory and activating receptors, even though functional data indicates that this receptor is involved in immune cell activation [52, 107]. In addition, KIR pseudogenes have been identified within the KIR locus [30]. Most KIRs are expressed as monomers on the cell surface. KIR mRNA can be alternatively spliced and KIRs display considerable allelic polymorphism, which adds to their diversity [108]. KIR2D molecules primarily recognize HLA-C ligands. The HLA-C α1 and α2 domains are contacted via amino acid residues located at the KIR D1and D2 domains [95].

LILRs are immune receptors expressed on the surface of subsets of NK and T cells, B cells and monocytes [109, 110]. They consist of either two or four extracellular Ig domains, a TM and a cytoplasmic tail containing ITIM-like motifs [110] or a short cytoplasmic tail together with a positive charge in their TM [111]. The latter LILR variety encompasses stimulatory receptor types of which one associates with the FcRγ chain signalling adaptor [112]. The LILR family of receptor loci are in close proximity to FcαR and KIR loci on human chromosome 19q13.2-q13.4. LILR1 and LILR2, both members of the LILR group 1, bind to HLA-A proteins, HLA-C proteins as well as HLA-G1. LILR1 and LILR2 display significantly less binding specificity than members of the KIR family, which recognize relatively restricted HLA subsets [111]. The LILR1 extracellular domains D1 and D2 contact their HLA ligand on two surfaces, the mainly non-polymorphic α3 domain and the highly conserved β2m molecule. Precisely, LILR1 contacts β2m via amino acid residues located at the membrane distal tip of D1 and the α3 subunit via residues located at the D1-D2 hinge region [94]. Interestingly, there is a group of receptors within the LILR family, which apparently do not bind MHC I complexes [94]. Termed Group 2 LILRs, these subsets exhibit a high amount of non-conserved amino acid substitutions within their MHC I contact site in comparison to group 1 LILRs. Group 2 LILRs may therefore recognize different ligands than MHC I, or they may have much lower affinity for the MHC I proteins. Ligand binding to the inhibitory group 1 receptors LILR1 and LILR2 inhibits early activation mediated by FcRs in NK cells via

activation of SHP-1 [31, 113]. In monocytes, LILRs may also regulate inflammatory responses and contribute to tissue homeostasis. Both, the LILR and the KIR receptor family genes are clustered in the human leukocyte receptor complex (LRC) on human chromosome 19. The LRC further encodes human FcαR and additional innate immune receptors [114]. In mice, there is also a designated LRC on chromosome 7 which encodes an Ly49 gene as well as PIRs among other immune receptor genes [114].

PIRs are Ig-like receptors that are expressed on mouse B cells, mast cells, macrophages and dendritic cells. PIR-A associates with FcRγ chain to mediate activatory signalling [115], whereas PIR-B inhibits receptor-mediated cellular activation [116].

The c-type lectin superfamily MHC I receptors are encoded in the NK complex in humans and mice. In humans, this receptor complex is located on chromosome 12p12-p13 and in mice on chromosome 6. MHC I receptors encoded in the human NK complex are the CD94/NKG2 heterodimers. The invariant CD94 molecule in these complexes is linked to a member of the NKG2 family glycoprotein via a disulfide bond [117-119]. The NKG2 family of receptors consists of four genes, and receptors NKG2A, NKG2C, NKG2E display diversity in their extracellular and intracellular domain structures, which could confer differential ligand binding and altered intracellular signalling capacities [120, 121]. CD94 lacks a cytoplasmic tail, and it is thought to act as a chaperone for NKG2 proteins, allowing the surface expression of many NKG2 receptors only in the form of CD94/NKG2 dimers [117]. NKG2D is expressed on NK and T cells and has an arginine residue in its TM. This residue allows for non-covalent association with signalling adaptor DAP10 containing an aspartic acid residue in its TM. The NKG2D receptor assembles as a homodimer with two DAP10 signalling dimers into a hexameric structure [122]. NKG2D interacts with a non-classical MHC I molecule (MICA) [123], which has been associated with tumour cells [17, 124]. Therefore, NKG2D-DAP10 mediated cytotoxicity may be a key player in the clearance of stressed/damaged cells and tumour cells.

Ly49 receptors are also lectin-like receptors and form disulfide bonded homodimers on the surface of NK cells. On T cell subsets, these receptors are expressed as inhibitory and stimulatory receptor types [125, 126]. These receptors are encoded within the mouse LRC and NK complex. Ly49 receptors regulate cell mediated cytotoxicity and release of chemokines and cytokines upon binding of MHC I or MHC I-like products encoded by viruses [125]. Ly49 contacts the α1 and/or α2 domain of the MHC I complex and requires β2m for binding [127, 128]. The kind of peptide loaded onto the peptide binding groove of the alpha chain does not affect Ly49 receptor ligand interactions [128, 129].

MHC I molecules exist throughout vertebrates [130], and components of the TCR complex involved in MHC I recognition by T cells have been described for many non-mammalian vertebrates including chickens [131-133], amphibians [134] and fish [135, 136]. However, the innate immune receptors that recognize MHC I molecules have yet to be identified in fish, amphibians and birds.

2.3 Channel catfish represents a unique vertebrate model for comparative immunological studies

The family of North American freshwater catfishes (*Ictaluridae*), which belong to the taxonomic group of bony fish (teleosts), consists of 45 species and is found from Guatemala to regions in Canada [137]. Unlike many other fish *Ictaluridae* do not have scales and have eight barbels near their mouth [137]. *Ictalurus punctatus*, named by polymath Constantine Samuel Rafinesque in 1818 in reference to its scattered black spots along the body, is a bottom feeder, and its diet primarily consists of aquatic insects, crayfish, molluscs, crustaceans and fish [138]. Interestingly, channel catfish cannot only sense food through taste receptors on their barbels but they also have a large number of taste receptors on their body surface. This phenomenon has made them an experimental model organism in studying taste receptor-amino acid interactions [139-141]. Channel catfish prefer cool, clear, deep water with sand, gravel or rubble bottoms as

habitat and usually seek protection underneath logs or rocks during the day. Spawning occurs in late spring or summer, and whether or not the fish migrate into rivers or moving waters depends on their habitat location. Seven to 15 days can pass between spawning and the fry leaving the nest when they swim to the surface to feed. The average size of a channel catfish is 36-53 cm and adult fish weigh on average between 0.9-1.8 kg [138]. According to Fisheries and Oceans Canada, channel catfish can be found in Canada in the St. Lawrence River and its tributaries, in southern Quebec, in the Ottawa River and tributaries, all the Great Lakes except Lake Superior and in many other lakes and rivers in Ontario and Manitoba. In the Great Lakes and the St. Lawrence River, the channel catfish is a relevant commercial fish species, a sport as well as food fish.

Immunological studies have long focussed on endothermic animals, such as humans and mice [142, 143]. Immunological mechanisms in ectothermic vertebrates remained relatively poorly understood, even though farmed fish are quite frequently affected by infectious diseases. This poses significant economical problems. Farmed fish are far more susceptible to infectious diseases because the predation of susceptible animals in this artificial system is missing and infections can easily spread to other fish, especially under crowded conditions. High stocking densities can lead to reduced water quality and prolonged stress, which can in turn result in immunosuppression. Also, under crowded conditions, fish are more likely to injure their fins and tails, another factor that contributes to disease and infection. To minimize loss of fish stock in aquaculture, adequate stocking density and water quality regarding levels of oxygen, ammonia and nitrite, for example, need to be provided [144]. There have also been attempts in breeding fish that are more resistant to disease [145, 146]. In this context, furthering the understanding of how fish fight disease is of great importance.

Parasites that specifically affect farm-raised channel catfish exposed to fish eating birds are nematodes and trematodes from infected birds. For example, *Bolbophorus* worm infections are associated with high channel catfish mortality and decreased production [147]. Enteric septicaemia is another relevant disease

affecting farm-raised channel catfish. This disease is associated with skin ulcers and petechial haemorrhages and the causative agent is the bacterium *Edwardsiella ictaluri* [148]. An even more frequently reported disease in channel catfish is visceral toxicosis, believed to be caused by *Chlostridium botulinum* infection [149]. Since the 1980s there have been substantial efforts to better understand the physiology and immunology of teleost such as the channel catfish. This process also had the purpose of bridging the understanding of immunological mechanisms between various organisms [150].

The successful development of *in vitro* culture methods allowed for the assessment of functions of specifically isolated catfish immune cells [151, 152]. Monoclonal antibodies have been generated against a variety of channel catfish proteins, including immune cell surface markers [152]. In the mid to late 1980s these tools allowed for functional *in vitro* studies on channel catfish immune cells, including mitogenic responses [153], mixed-leukocyte reactions [151], antibody production [154], interleukin production [155, 156] as well as cellular cytotoxicity [157]. The influence of mild stress on immunosuppression is another area of research that routinely uses channel catfish as an immunological model [158, 159]. Similar to mammals, bony fish have characteristic innate and adaptive immune systems [160]. Clonal lymphoid cell lines can be spontaneously developed from freshly isolated channel catfish peripheral blood leukocytes, and there are established T, B , monocyte and NK cell lines [161]. Antibody-dependent cell-mediated cytotoxicity (ADCC) in a channel catfish NK-like cell points to the redundancies between immunological processes in phylogenetically distant vertebrates such as mammals and bony fish [162, 163]. Moreover, catfish NK cells have been shown to kill allogeneic target cells independent of ADCC mechanisms [164]. This leads to the hypothesis that NK cell cytotoxicity in catfish may include receptor functions similar to those of KIRs and LILRs in allogeneic recognition by human NK cells [165]. The insights gained from using channel catfish as a non-traditional immunological model will benefit aquaculture in general and also will continue to provide insights into the evolutionary

conservation of the immune system. In this context such studies have allowed major advances the field of comparative immunology.

2.4 Channel catfish leukocyte immune-type receptors

2.4.1 Background

The generation and screening of channel catfish expressed sequence tag (EST) libraries led to the identification of three sequences that encode type I TM proteins with variable numbers of extracellular C2 Ig-like domains (Fig. 2.4). These three proteins vary in length but display high sequence similarity. Termed *Ictalurus punctatus* leukocyte immune-type receptors (IpLITR), these molecules have identical leader peptides. IpLITR1 and IpLITR3 have almost identical Ig domains D1, D2, D3 and D4. In comparison, the Ig domains D1 and D2 of IpLITR2 is 77.2% and 88.6% identical to those of IpLITR1 and IpLITR3 at the amino acid level. IpLITR1 contains four Ig domains, a TM and a long cytoplasmic tail with two ITIM motifs, an ITIM-like motif and an overlapping tyrosine based switch motif, whereas IpLITR2 and IpLITR3 contain three and six Ig domains, respectively, and a positive charge in their TMs contributed by a lysine residue. These receptors also lack intrinsic signalling motifs on their short cytoplasmic tails. There is a membrane distal and membrane proximal ordering to the IpLITR extracellular Ig domains. The membrane distal domains D1 and D2 are similar, whereas other IpLITR Ig domains share less sequence homology. For instance, the composition of the membrane proximal domain D3 of IpLITR2 shares very little identity (17.9%-39.3%) with other IpLITR Ig-like domains [28].

Southern blot analysis of digested genomic DNA taken from catfish siblings revealed that the IpLITR gene complex is polygenic and polymorphic. There is a number of hybridizing bands for each individual animal, and there is a multitude of banding patterns between siblings. IpLITR homologues have also been identified in zebrafish (*Danio rerio*), and genome mining demonstrated that

five separate contigs containing IpLITR homologues are on zebrafish chromosomes 3, 7 and 8. The notion that IpLITRs genes are also encoded on multiple, independently segregating homologous loci suggests that the various IpLITR genes are a result of multiple gene duplication and translocation events from a common ancestral gene [28].

Northern blot and PCR analyses showed high levels of expression of IpLITRs in the kidney (the fish hematopoietic organ) and low levels in other organs such as gills, heart, muscle, thymus, intestine and the spleen. The banding pattern in the reverse transcriptase polymerase chain reaction (RT-PCR) results revealed that there are several IpLITRs expressed in these organs. Not all organs appear to express the same types of IpLITR molecules. This trend is even more obvious when analyzing RT-PCR results obtained from channel catfish leukocyte cell lines, all of which seem to express various types of IpLITRs. Interestingly, cytotoxic T cells as well as NK cells exhibit a pattern of differential expression of IpLITRs over time following alloantigen stimulation with irradiated 3B11 cells. Importantly, stimulatory and inhibitory IpLITR types are co-expressed within catfish tissues as well as cultured cell lines. These findings suggest a vital role of these receptor molecules in the control of catfish immune cell effector functions [28].

Sequencing of these various RT-PCR products from tissues and cell lines resulted in the identification of several additional IpLITR-types whose denomination is based on the previously discovered IpLITR prototypes IpLITR1, IpLITR2 and IpLITR3. These various molecules differ in their number of extracellular Ig domains and, in the case of IpLITR1 homologues, have different cytoplasmic ITIM-containing tails [28].

Amino acid analyses revealed that IpLITRs are distantly related to well-studied mammalian immune receptor families such as FcRs and receptors encoded in the LRC, such as KIRs and LILRs. They are also distantly related to PIRs, CHIRs and frog Fc like immune receptors (XFL) as well as human Fc receptor like molecules (FCRL). Those receptor families are located on different

chromosomes of the genome and serve a wide array of functions, be it in self/non-self recognition (KIRs, LILRs, PIRs) or as Fc receptors (FcRs, CHIRs) [28]. Even though there is a relationship between the extracellular domains of IpLITRs and these receptor families, they may not be functional homologues. For instance, FCRLs are related to classical FcRs but do not appear to bind Igs [166, 167]. This points to the possibility that some immune receptors from different animal species, while related, have evolved to recognize different ligands. The discovery that PIRs, which are also related to FcRs, bind mouse MHC I and β2m [99], has prompted the question whether PIR related immunoregulatory receptors, such as IpLITRs may also bind MHC I [165]. Functional studies of IpLITR signalling and the identification of their ligands have a great potential to provide further insights into the common origin and evolutionary history of innate immunoregulatory receptor families.

2.4.2 IpLITR inhibitory and stimulatory signalling potential

Several IpLITRs contain ITIM motifs in their cytoplasmic tail, suggesting that they may inhibit immune cell responses. Others have charged TMs and short cytoplasmic tails indicating a stimulatory signalling potential [28]. Whether different IpLITR-types have the ability to recruit inhibitory phosphatases or assemble with ITAM-bearing adaptor molecules therefore poses an important research question.

To examine whether inhibitory IpLITR-types could recruit phosphatases to their intracellular ITIM motifs, IpLITR chimeric molecules were generated. These chimeric receptor molecules consist of the extracellular Ig domains and TM of KIR2DL3 and cytoplasmic tail of the putative inhibitory IpLITR receptors. The KIR2DL3 extracellular domain allows for detection with a mononclonal DX27 antibody. This is an attractive strategy, as there are no specific antibodies available against IpLITRs. Several versions of this fusion receptor were generated: some containing the native IpLITR1.2a or IpLITR1.1b cytoplasmic

sequence, others containing truncated versions of IpLITR cytoplasmic tails lacking ITIM motifs. These IpLITR-KIR constructs were co-expressed with FLAG epitope-tagged recombinant zebrafish SHP-1 and/or SHP-2 proteins in HEK 293T cells. The chimeras were immunoprecipitated after pervanadate treatment to induce receptor ITIM tyrosine phosphorylation. Only those IpLITR fusion proteins containing the predicted ITIM motifs were able to recruit zebrafish SHP-1 and SHP-2 following tyrosine phosphorylation. These results support the notion that inhibitory IpLITR-types can indeed recruit teleost inhibitory phosphatases via phosphorylated tyrosines encoded within their ITIMs. This represents a first step in an inhibitory signalling cascade and further studies on their role in immune effector roles are currently underway [168].

To investigate whether a putative stimulatory IpLITR-type recruits one or more teleost signalling adaptors is one of the objectives of this thesis, and the findings of these studies are the subject of Chapter 4.

2.4.3 IpLITR predicted binding partners

Many different forms of stimulatory and inhibitory IpLITR types with variable numbers of extracellular domains are expressed in catfish lymphocytes. However, there is no information regarding their functional significance. Alterations in expressed immune receptor Ig domains are observed in the FcR family, where high affinity binding is associated with the presence of three extracellular Ig domains and low affinity FcRs have only two extracellular Ig domains [86, 169]. Therefore, variations in Ig domain composition may affect IpLITR ligand affinity. It is also likely that the length of the receptor, reflected by the number of extracellular domains, influences accessibility to cellular ligands. Human LRC-encoded immune receptors recognizing MHC I ligands contact these ligands through different regions in their extracellular domains. The four Ig domain-containing LILRB1 uses residues on the distal tip of D1 to contact the α3 domain and residues located at the hinge of D1D2 for receptor ligand interactions at the

β2m site [94] (Fig. 2.5). The shorter two-domain KIR2DL1, on the other hand, interacts with the polymorphic α1 and α2 domains of its HLA ligand through its D1 and D2 domains [95]. Therefore, IpLITR Ig domain variations could affect binding affinity and allow for different modes of receptor-ligand interactions [165].

Interestingly, all IpLITRs sequenced to date contain similar membrane distal domains D1 and D2, which may be involved in ligand binding. Sequence comparison of IpLITRs to MHC I receptors resulted in the identification of a putative MHC I binding site [165]. This site was very similar to the MHC I α3 binding site of LILRB1 but very distinct from the KIR2DL1 ligand binding site. Within the distal tip of some of the IpLITR domains D1, there are five key residues conserved or identical with those of LILRB1 that contact the α3 domain of MHC I. An additional tyrosine within the first Ig domain was conserved between many of these representative IpLITRs and human LILRB1. The alignment of the human LILR family with IpLITR representatives further revealed several nonconserved substitutions within the putative IpLITR ligand binding site. This phenomenon is also present within the group 2 of LILRs that have not been shown to bind MHC I alleles. Therefore, some IpLITRs may bind the α3 contact side of MHC I, whereas others may have variable affinity for MHC I or bind different ligands altogether. Moreover, some β2m binding residues between LILRB1 and IpLITR representatives are conserved, even though a putative β2m binding site within the IpLITR D1 D2 domains is less obvious than a putative α3 binding site. Only four of the twelve contact residues are conserved in some IpLITRs when compared to LILRB1 contact residues. One key LILRB1 β2m binding residue was changed to a proline in many IpLITRs. Additional non-conserved amino acid substitutions are present also within the IpLITR β2m binding site. In 3-dimensional modelling of IpLITRs with LILRB1, the conserved binding residues of IpLITRs locate to similar positions as in LILRB1. The α3 contact residues are positioned at the distal tip of D1 and the β2m contact site is

situated at the predicted IpLITR D1D2 hinge. Some of the IpLITR 3-D structures are predicted to contain a 3_{10}-helix within their D1 domain, a hallmark feature of the MHC I binding receptor LILRB1 [94, 170, 171]. Other IpLITRs have a predicted C' strand at this position, a characteristic also observed in a non-MHC I binding LILRs [172]. The presence of this C' strand is associated with reduced or lacking MHC I affinity in LILR group 2 members [172]. This correlation, again, points to the hypothesis that some IpLITRs may bind catfish MHC I molecules whereas others may not. Notably, all these trends are based on comparative homology modelling. Only the analysis of IpLITR crystal structures will allow more definitive answers on the exact folding and location of residues in IpLITR D1 D2 domains [165].

When studying and comparing conserved contact amino acid residues between receptors of evolutionarily removed species, one also has to consider the conservation of contact residues within the ligands in these species. In the case of β2m, about half of the residues contacted by LILRB1 D1 and D2 domains are also present in catfish β2m. When mapping these residues onto the predicted catfish β2m 3-D structure, they locate to similar positions as the contact residues in human β2m. Two of the six residues within the α3 domain of HLA-A2 contacted by LILRB1 are also present within four representative catfish MHC I molecules. If IpLITRs bound catfish MHC I it would then be expected that additional contact residues within the α chain and β2m are involved in the IpLITR-MHC I interaction [165].

Despite their distant phylogenetic relationships with mammalian FcRs, there is no evidence of a conserved Fc-binding site within any IpLITR D1 and D2 domains examined to date [165]. In addition, a bona fide catfish FcR that recognizes catfish IgM is encoded as a single copy gene that is not related to any IpLITR family members [61]. However, it is still possible that additional catfish FcRs may be encoded within the IpLITR genes. In support of this hypothesis, a recently identified Fc-binding receptor in chicken called CHIR-AB1, stems from a diverse family of immune receptors predicted to be orthologues to mammalian

LRC-encoded receptors and not FcRs or FCRLs [25, 173]. Therefore, the possibility that some IpLITRs bind catfish immunoglobulins or other ligands cannot be excluded and is under investigation.

2.5 Summary

Immune receptors are mediators of cellular effector responses in all vertebrates. They are involved in recognition of pathogens and infected or otherwise unhealthy cells; they bind antibodies and are crucial for self/non-self recognition. The complex actions of these receptor networks determine successful clearing of infectious agents, eradication of tumour cells, and in the case of tissue transplantations, immune rejection and/or tolerance. Disturbances within these networks can be the underlying cause for autoimmune diseases [174-176]. It is therefore not surprising that such vital functions exhibit a significant degree of conservation between animal species.

Innate immune receptor signalling is highly conserved among vertebrate species [177]. The extracellular domains of innate immune receptors form the ligand binding domains of the receptors. Inhibitory receptors signal via intracellular ITIM motifs and stimulatory receptors often associate with a signalling adaptor carrying ITAM motifs. The signalling cascades set off via recruitment of enzymes to these sites can result in killing of target cells, sparing of target cells, phagocytosis or receptor-ligand complex internalization followed by antigen display.

IpLITRs represent a novel immune receptor family from channel catfish. A large array of these receptors, including inhibitory and stimulatory types, is expressed in channel catfish leukocytes. These molecules are distantly related to diverse families of other vertebrate innate immune receptors that have various functions and are located on different chromosomes within their host genomes [28]. The functional characterization of IpLITRs will yield important insights into the evolution and functional conservation of innate immune receptors among

vertebrates. Additionally, the understanding of their role as regulators of immune effector functions in fighting diseases and in response to toxins will allow for applications in the fields of aquaculture and environmental toxicology.

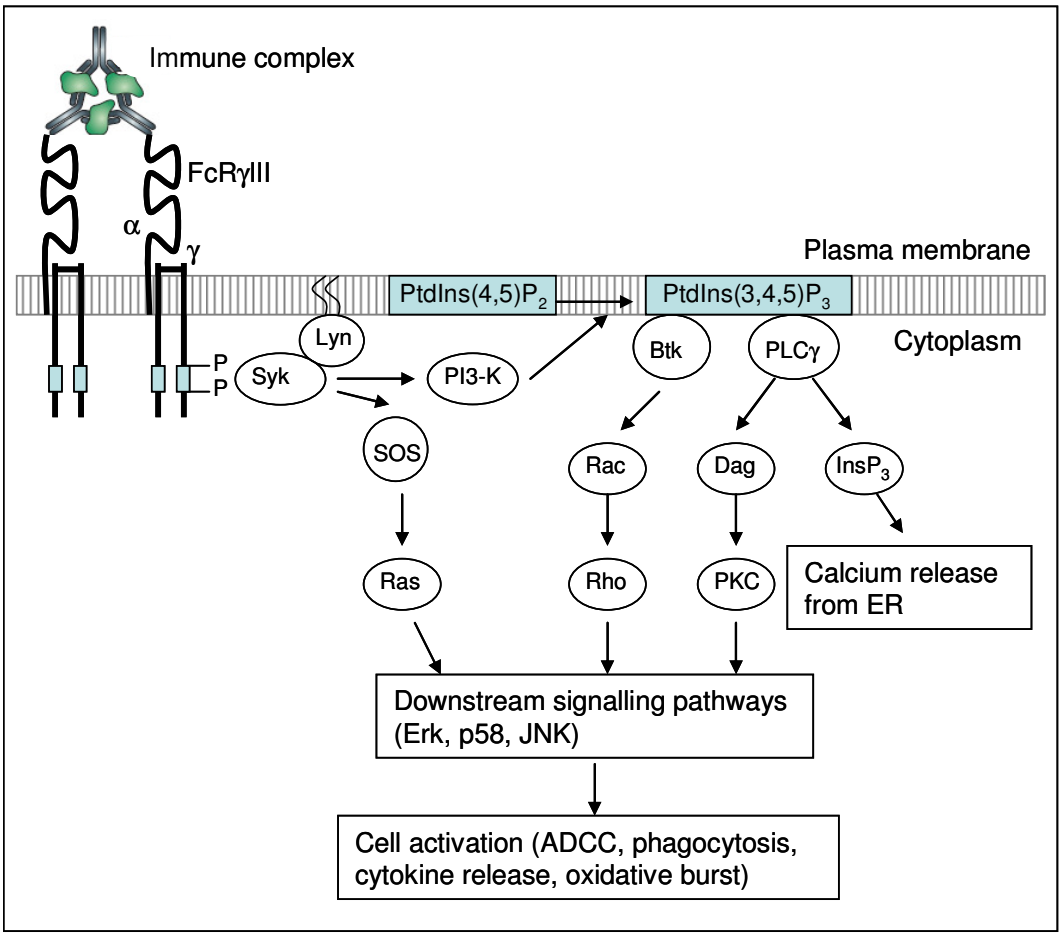


Figure 2.1. FcRγ chain dimers trigger signalling pathways in innate immune cells (adapted from [8])**.** Immune complex binding to the innate immune receptor FcRγRIII results in receptor crosslinking. This induces the phosphorylation of tyrosine residues of the ITAM within the receptor associated signalling adaptor FcRγ chain via Src family kinase Lyn. The SH2-binding domain containing enzyme Syk is then recruited to the newly created docking sites on the signalling adaptor and sets off signalling cascades. These cascades include signal transducing enzymes such as PI3-K and son of sevenless homologue (SOS). The generation of phosphatidylinositol-3,4,5-trisphosphate (PtdIns(3,4,5)P3) recruits Bruton's tyrosine kinase (Btk) and PLCγ, which leads to activation of downstream kinases and the release of calcium from the endoplasmic reticulum (ER).

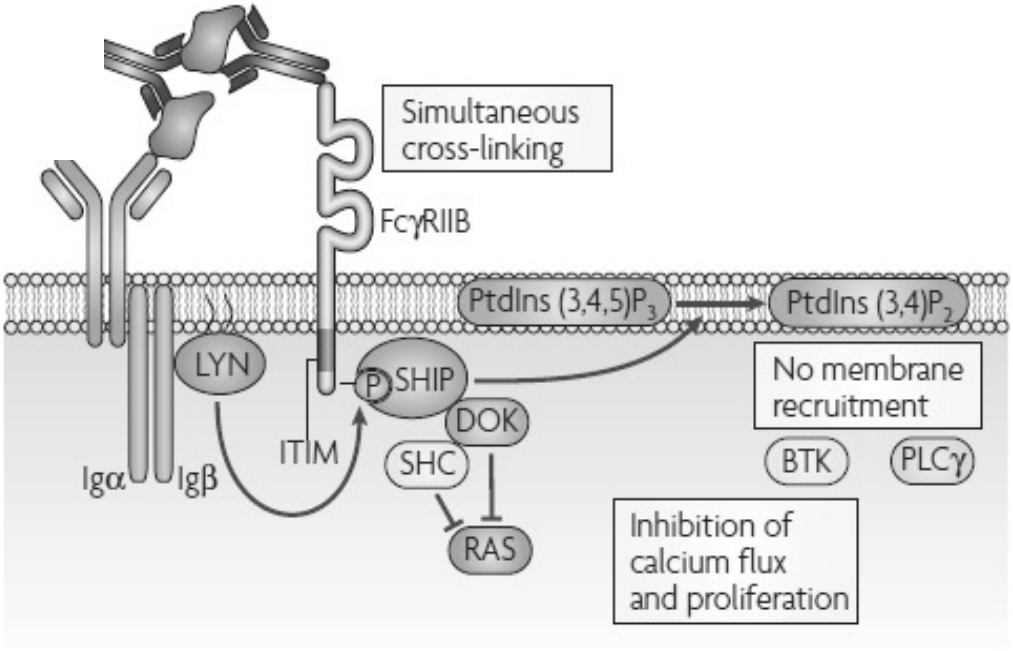


Figure 2.2. Immune receptors carrying intracellular ITIMs inhibit immune cell activation (adapted from [8])**.** Upon ligand binding to the inhibitory receptor – in the case of FcγRIIB: crosslinking of activatory and inhibitory receptor – Src family kinases such as Lyn phosphorylate tyrosine residues within the inhibitory receptor ITIM, which then create a docking site for inhibitory phosphatases such as SHIP. SHIP then hydrolyses phosphatidylinositol (3,4,5)-trisphosphate ($PtdIns(3,4,5)P_3$) into phosphatidylinositol-4,5-bisphosphate ($PtdIns(4,5)P_2$), which ultimately inhibits recruitment of pleckstrin homology (PH)-domain containing proteins such as Btk and PLCγ. Furthermore, SHIP recruits adaptor proteins such as Dok that sequester proteins involved in activatory signalling pathways away from their activating complexes and thereby inhibit signalling cascades involving Ras [178]. While this example displays inhibitory signalling in B cells, the inhibitory signalling events in innate immune cells are analogous to those shown here.

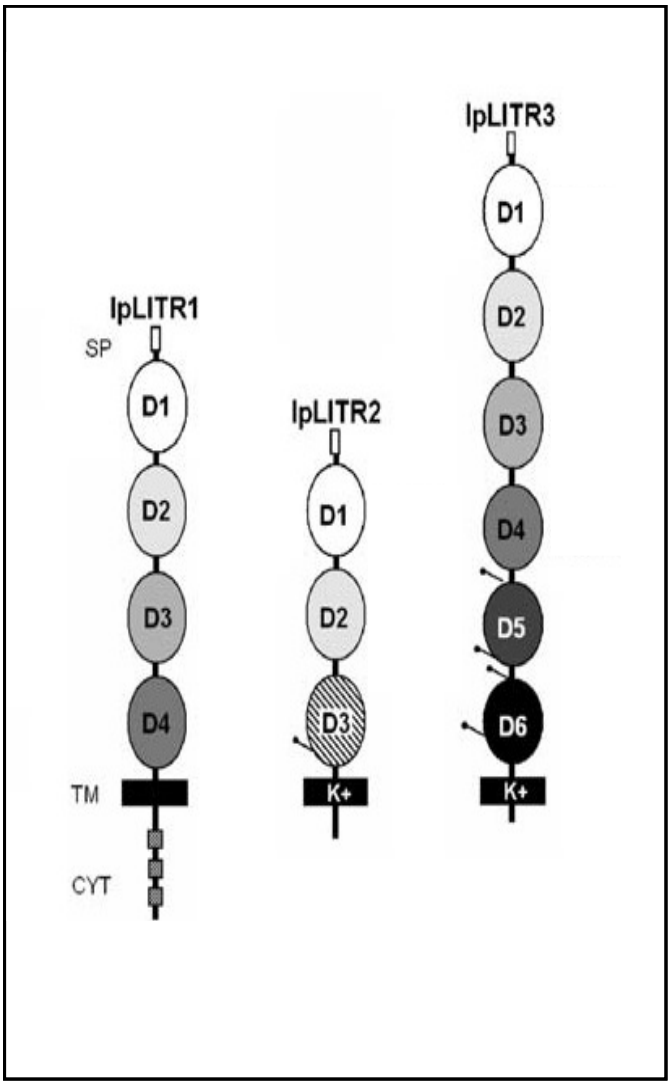


Figure 2.3. Schematic representation of IpLITR prototypes IpLITR1, IpLITR2, and IpLITR3 (adapted from [28])**.** The predicted signal peptide (SP), Ig domains, TM, and cytoplasmic tail (CYT) are indicated. ITIM-like motifs are shown as boxes, N-linked glycosylation sites are marked as ballpoint lines. Individual IpLITR domains are shaded according to their relatedness between IpLITRs.

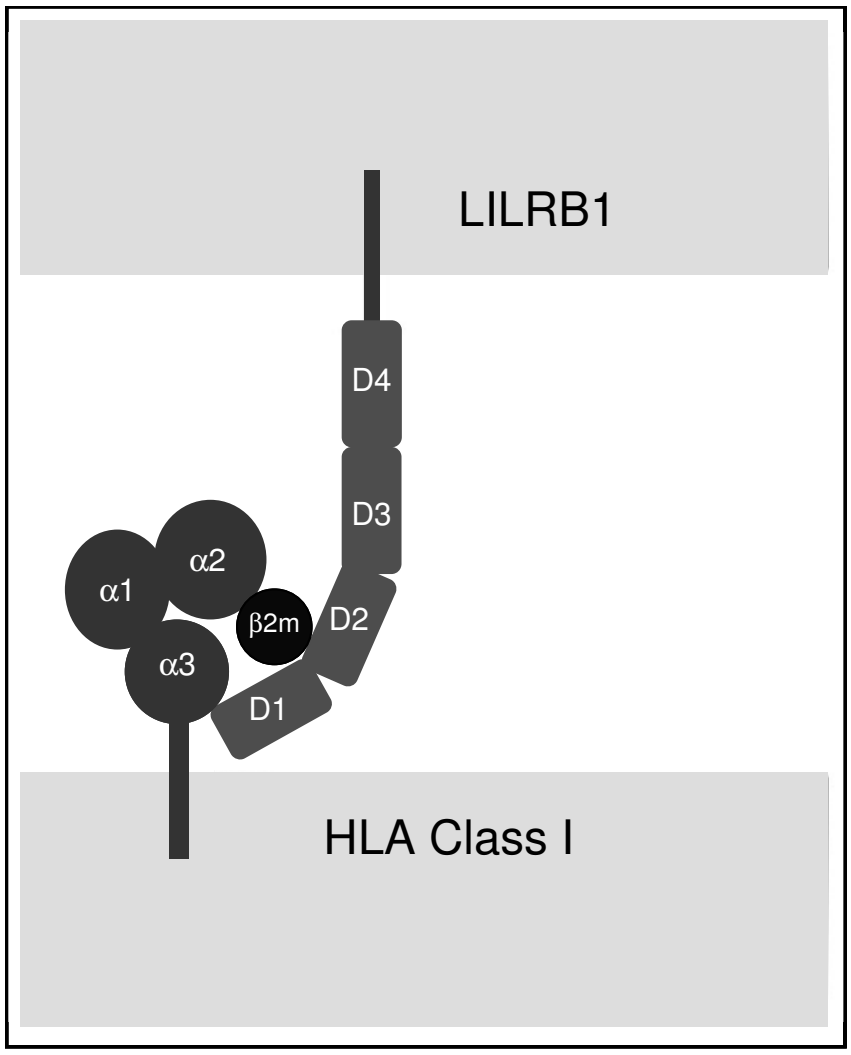


Figure 2.4. IpLITRs containing a putative MHC I binding site could contact MHC I complexes in a similar manner as LILRB1 (adapted from [31])**.** LILRB1 binds HLA class I complexes at the tip of its membrane distal domain D1 and at the D1-D2 hinge. The LILRB1 D1 tip binds residues in the α3 domain of HLA and the D1-D2 hinge contacts residues within the β2m subunit of the HLA class I complex.

CHAPTER 3
MATERIALS AND METHODS

3.1 Cell lines

HEK 293T cells are derived from HEK 293 cells by transformation with large T antigen of simian virus 40 (SV40) that inactivated pRb and were kindly provided by Dr. Burshtyn (Dept. Medical Microbiology and Immunology, University of Alberta). HEK 293 cells, in turn, are Human Embryonic Kidney cells, most likely derived from a neuronal lineage cell [179] transformed with sheared fragments of adenovirus type 5 DNA by Graham et al. in the 1970s. [180].

The 3B11 and 1G8 channel catfish B cell lines are a kind gift from the laboratory of Dr. Wilson (University of Mississippi Medical Centre). These cell lines were generated from an outbred catfish by mitogen stimulation [71].

3.2 Cell Culture

The HEK 293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM)/High Glucose (HyClone) supplemented with 2 mM L-glutamine (Gibco), 100 U/mL penicillin (Gibco), 100 µg/mL streptomycin (Gibco), 1 mM sodium pyruvate (Gibco), 1% MEM non-essential amino acid solution (Gibco), and 10% heat-inactivated fetal bovine serum (characterized; Hyclone) at 37℃ in 5% CO_2. Prior to use, culture media was filter sterilized using 0.22 µm filter units (Corning). For channel catfish MHC I expression studies, transfected HEK 293T cells were cultured in the same complete DMEM medium but some culture subsets were incubated at 27℃ for 24 h and 5% CO_2 following a 72 h incubation at 37℃.

3B11 cells and 1G8 cells were cultured in AL-4 medium containing equal amounts of AIM-V (GIBCO) and L-15 media (SIGMA) supplemented with 1 µg/ml $NaHCO_3$, 50 U/ml penicillin, 50 µg/ml streptomycin, 20 µg/ml gentamicin,

50 μM 2-β-mercaptoethanol, and 4% heat inactivated, pooled, normal catfish serum [161]. The catfish serum was kindly provided by Dr. Wilson's laboratory (University of Mississippi Medical Centre). The cultures were incubated at 27℃ and 5% CO_2.

3.3 Antibodies

Antibodies used for flow cytometry were: mouse anti-hemagglutinin (HA) monoclonal antibody (mAb) clone HA.C5 (Cedarlane Laboratories Ltd.), mouse anti-FLAG mAb M2 (Stratagene), and goat anti-mouse IgG (H + L)-phycoerythrin (PE) (Beckman Coulter), chicken anti-channel catfish β2m polyclonal antibody (pAb) (kindly provided by the laboratory of Dr. M. Wilson), mouse anti-chicken-PE (Beckman Coulter), mouse anti-chicken biotin (Beckman Coulter), goat anti-mouse IgG3-PE (Beckman Coulter).

Antibodies used in Western blots were: horse-radish-peroxidase (HRP)-conjugated goat anti-HA polyclonal antibody (GenScript Corp.), anti-FLAG M2 mAb HRP-conjugated (Sigma–Aldrich), chicken anti-channel catfish β2m pAb and HRP-conjugated mouse anti-chicken antibody (Beckman Coulter), goat anti-mouse IgG3-HRP (Beckman Coulter), goat anti-mouse IgG(H+L)-HRP (BioRad), rabbit anti-mouse IgG, Fc (US Biological) and goat anti-rabbit IgG(H+L)-HRP (BioRad) as well as goat anti-mouse IgG(H+L)-AP (BioRad).

3.4 Generation of epitope tagged IpLITR proteins

Putative stimulatory IpLITR representative IpLITR 2.6b (DQ860171.1) was cloned from catfish leukocyte cell lines [28, 61, 165](Stafford et al., 2006, 2007). The various cDNAs were originally cloned into the pCR4-TOPO vector (Invitrogen) and provided as a kind gift from Dr. Norman Miller (Univ. Mississippi Medical Center). In order to express IpLITR 2.6b on the surface of HEK 293T cells the receptor DNA was amplified from 10 μg of pCR4TOPO in a

PCR reaction using 0.4 U of Phusion High-Fidelity DNA polymerase (Finnzymes) in 20 µL reactions according to manufacturer's recommended instructions with pDisplay_IpLITR_SmaI_Fwd and pDisplay_IpLITR_SalI_Rvs primers adding SmaI and SalI sites (Table 1). Cycling parameters were as follows; 40 s at 98℃, 30 cycles of 98℃ for 20 s, 64℃ for 25 s, 72℃ for 30 s, and a final extension step for 7 min at 72℃. The PCR reaction was separated on a 1.0% TAE-agarose gel and visualized by staining with ethidium bromide solution (50 µg/L). The PCR product was gel purified (Qiagen Gel Extraction Kit) and then subcloned into pJET1.2/blunt using the blunt-end protocol (Fermentas). Then 25 µL of *E.coli* NEB 10-beta (New England Bioloabs) were transformed with 5 µL of the cloning reactions and positive clones were identified by colony PCR using the cloning forward and reverse primers. Then, 8 mL liquid LB culture media containing 100 µg/mL ampicillin were inoculated with colonies from positive clones and grown over night at 37℃ on a rotary shaker at 225 rpm. Plasmids were isolated from these *E.coli* cultured using the Qiaquick Miniprep kit (Qiagen) according to manufacturers instructions and 2 µg of purified plasmids were subjected to restriction digest with 10 U SmaI and SalI (Fermentas) followed by gel purification. The insert was then used in a ligation reaction with SmaI-SalI digested purified pDisplay™ (Invitrogen) vector with T4 DNA ligase (Fermentas) and a 2.5 h incubation at 25-8℃ followed by an overnight incubation alternating between 4-8℃ and 16℃ every 45 min. Bacterial transformations and identification of positive cones were performed as above, so was the plasmid preparation from positive clones.

The IpLITR 2.6b sequence in pDisplay™ was verified by sequencing with the cloning primers to ensure the proper insertion of the receptor cDNA into pDisplay™ without base pair changes or frame shifts. All sequencing was performed at the Molecular Biology Services Unit in the Department of Biological Sciences (University of Alberta) on an ABI 3730 DNA sequencer.

3.5 Generation of N-terminal FLAG tagged teleost adaptor proteins

ITAM-containing adaptors *Ictalurus punctatus* (Ip) FcRγ (AF538721.1), IpFcRγ-L (AF543420.1), IpCD3ζ-L (DQ114900.1), IpDAP12 (EU925386.1), IpDAP10 (DQ114898.1), *Danio rerio* (Dr) FcRγ (NM 001100106.1), DrFcRγ-L (NM 001100156.1), DrCD3ζ (NM 001100157.1), DrCD3ζ-L (NM 001100107.1), DrDAP12 (NM 001100103.1), and DrDAP10 (NM 001100102.1) were used in these experiments. Total kidney RNA was isolated from catfish and zebrafish tissues using TriZol reagent (Invitrogen) according to manufacturer's instructions and the RNA (1 μg) was converted into cDNA using Oligo(dT)18 primer and 40 U of MMuLV reverse transcriptase (Fermentas). The resulting cDNAs were cloned into the p3xFLAG-CMV-9 expression vector (Sigma–Aldrich) using the procedure described above (Chapter 3.4) with primers adding HindIII (foward) and BamHI (reverse) sites (Table 3.1). All constructs were sequenced using the cloning primers prior to use in transfection studies.

3.6 Generation of soluble IpLITR-Fc fusion proteins

To generate soluble fusion proteins as staining agents to screen target cells for ligand expression the D1 and D2 domains from putative stimulatory IpLITR 2.0 (NCBI accession number: AY885644.1), 2.3a (DQ860176.1), 2.6b (DQ860171.1) and 1-3 (AY885645.1) were amplified from the pCR4-TOPO vector (Invitrogen) into which the IpLITRs were originally cloned. The primers used were pFuse_IpLITR_EcoRV_Fwd, pFuse_IpLITR_BglII_Rvs or pFuse_IpLITR_NcoI_Rvs (Table 3.1) and added an EcoRV site to all resulting PCR products and a BglII site provided by the Rvs primer to the IpLITR 2.0, 2.3a and 2.6b constructs. The IpLITR 3.0 D1 and D2 domains were amplified using the same forward primer but a reverse primer adding a NcoI site to the construct. The subcloning was done as above, except that *E.coli* FastMedia™ zeomycin media (Invivogen) were used as for selection of bacterial cloning into the

destination pFUSE-mIgG3-Fc2 vector, which also occurred via the appropriate restriction digest. The pFUSE-mIgG3-Fc2 vector adds a mammalian interleukin 2 (IL2) secretion signal N-terminally and C-terminal human Cγ2 and Cγ3 domains of IgG3.

Another set of constructs was made cloning the IpLITR-Fc fusion constructs into the pDipsplay™ vector adding an HA-tag N-terminally to the fusion protein as well as an Ig κ leader peptide for secretion of the fusion proteins into the cell culture supernatant. These constructs were made starting with a PCR reaction of the IpLITR-Fc constructs in the pFUSE-mIgG3-Fc2 vector. The standard sub-cloning method described earlier (see 3.4) was applied, again, using primers pDisplay_IpLITR_SmaI_Fwd and pFuse-pDisplay_IpLITR_SalI_Rvs adding SmaI (Fwd) and SalI (Rvs) sites to the PCR product. All final constructs were confirmed via sequencing using the cloning primers.

3.7 Generation of chimeric IpLITR-CD3ζ proteins

To generate chimeric IpLITR-CD3ζ proteins, IpLITR 1-3-Fc and 2.6b-Fc constructs were cloned into the BWZ type I vector which was kindly supplied by Dr. J. Carlyle (University of Toronto, Sunnybrook Research Institute). To generate constructs without HA epitope tag the constructs were cloned from the IpLITR-Fc constructs in pFUSE-mIgG3-Fc2 using primers pFuse-BWZ_XhoI_Fwd and pFuse-BWZ_NotI_Rvs adding XhoI and NotI sites to the PCR products. The cloning and subcloning procedures were as described above (see 3.4) with the exception that the insert for the destination BWZ type I vector was released from pJET1/2blunt using a sequential digest starting with a NotI digest followed by a XhoI digest using Fastdigest® enzymes (Fermentas) and buffers according to supplier's instructions.

Another set of constructs was made cloning HA-IpLITR-Fc fusion constructs containing the D1 and D2 domains of IpLITR 1-3 and 2.6b from the pDipsplay™ vector into the BWZ type I vector. The cloning technique applied

was the same as for untagged IpLITR-CD3ζ constructs. The primers used were pFuse-pDisplay_BWZ_NotI_Fwd and pFuse_BWZ_XhoI_Rvs.

All final constructs were confirmed via sequencing using the cloning primers as well as BWZ_IpLITR_2.6b_internal_Rvs and BWZ_IpLITR_1-3_internal_Rvs. The primers pFuse-pDisplay_BWZ_internal_Fwd and D4_mFc_pFuse_BWZ_Fwd were used to sequence through and beyond the Fc domains and ensure no frame shifts occurred prior to the TM and CD3ζ sequence.

3.8 Generation of epitope tagged catfish MHC I α chain

RNA was isolated from the channel catfish 3B11 B cell line: $4.2x10^6$ cells were used for RNA extraction with 500 µL of TriReagent (Sigma) according to supplier's instructions. For cDNA synthesis 2 µg of RNA were used in a 20 µL reaction as described above (see 3.5). Then, the primers pDisplay_IpMHCIalpha_SmaI_Fwd and pDisplay_IpMHCIalpha_SalI_Rvs designed for the catfish MHC I alpha chain allel Icpu-UBA*01 (AF053547.1) with an added SmaI and SalI site were used in a PCR reaction as described above (see 3.4). Cycling parameters were as follows; 40 s at 98°C, 30 cycles of 98°C for 20 s, 64°C for 25 s, 72°C for 1 min, and a final extension step for 7 min at 72°C. The subcloning and the final cloning technique into the pDisplay™ was as described above (see 3.4).

To generate a catfish MHC I alpha chain construct containing an N-terminal FLAG tag, the MHC I α cDNA was amplified from the subcloning vector using primers pCMV-9_IpMHCIalpha_NotI_Fwd and pCMV-9_IpMHCIalpha_EcoRV_Rvs adding appropriate restriction sites for cloning into p3xFLAG-CMV-9 as described above (see 3.4).

The final constructs were sequenced as described above (see 3.4) with the primers used for cloning as well as with T7_Fwd and BGH_Rvs for HA-tagged alpha chain in pDisplay.

3.9 Generation of epitope tagged and untagged catfish β2m

To clone catfish β2m the same cDNA sample as prepared for the cloning of MHC I α chain was used (see 3.8). The cDNA was subcloned as described above with the final cloning vector target being into p3xFLAG-CMV-9 as described above (see 3.8). The primers used for this procedure were pCMV-9_Ipb2m_HindIII_Fwd and pCMV-9_Ipb2m_BamHI_Rvs.

In order to generate untagged catfish β2m constructs for expression in HEK 293T cells the β2m cDNA from the subcloning vector was amplified with appropriate primers pFUSE_Ipb2m_EcoRV_Fwd and pFUSE_Ipb2m_BglII_Rvs to allow for cloning into the pFUSE-mIgG3-Fc2 as described in 3.6. The constructs were confirmed by sequencing using the cloning primers.

3.10 Cellular transfections

Transfections of HEK 293T cells for surface expression and association studies of IpLITRs and signalling adaptors as well as for MHC I expression were performed with cells seeded in six-well tissue culture plates (Costar). To ensure that cultures had >95% viable cells prior to seeding for transfection experiments, cell viability was examined using Trypan blue staining. Then, 7.5 x 10^5 cells were seeded in 2 mL DMEM/10% FBS per well and incubated 48 h prior to transfection with plasmid DNA. For each expression construct, DNA was first diluted in 190 µL of serum-free DMEM and then 4 µL of TurboFect in vitro transfection reagent (Fermentas) was added. For expression and association studies of IpLITRs and teleost adaptors 0.5 µg of IpLITR plasmid DNA and/or 1.0 µg of adaptor plasmid DNA was used. For single transfections of soluble Fc fusion proteins 1µg of the plasmid was used per cell culture well, and in expression and association studies of catfish MHC I 1 µg of plasmid of the catfish MHC I α chain containing expression construct and/or 1 µg of the catfish β2m construct was used. Samples

were gently mixed and incubated for 20 min at room temperature. The DMEM-plasmid-TurboFect mixture was then evenly layered onto the cells, followed by incubation for 48 h at 37℃ to allow for protein production. For MHC I expression studies some subsets of transfected cells were incubated for 24 h at 36℃ to allow for some protein production before transferring the cultures to the 27℃ incubator for another 24 h in order to promote surface expression of the complex.

For the production of Fc-fusion proteins, 500,000 HEK 293T cells were seeded into 12 mm tissue culture dishes (Costar) and incubated until the cell cultures reached 75% confluency (48 h). Then, the cells were transfected with 6 μg HA-IpLITR 1-3-Fc or HA-IpLITR 2.6b-Fc expression constructs, 12 μL Turbofect transfection reagent and 580 μL serum-free DMEM in a procedure as described above.

3.11 Flow cytometry of HEK 293T cells

The HEK 293T cell culture media supernatant was removed from each culture well 48 h post-transfection. All subsequent steps were conducted in a gentle manner to minimize loss of cells. The cells were then washed with 2 mL of sterile Dulbecco's phosphate buffered saline (D-PBS) containing 2mM of EDTA. The D-PBS was aspirated followed by addition of 500 μL of 0.25% Trypsin–EDTA (Gibco) to each well. The plate was agitated until cells detached. One millilitre of DMEM–10% fetal bovine serum (FBS) was added and 500 μL aliquots (1×10^6 cells) placed in 1.5 mL Eppendorf tubes for staining. Cells were centrifuged at 4℃ (400×g for 8 min), supernatants aspirated and 1.4 mL of ice-cold antibody staining buffer (ASB) (D-PBS, 0.05% NaN_3, 1% bovine serum albumin (BSA)) added. The cells were centrifuged again and the supernatants aspirated. After disruption of the cell pellets 1μg of primary antibody (anti-HA mAb clone HA.C5, anti-FLAG mAb M2 was added or anti-channel catfish β2m pAB) diluted in 50 μL of ASB. Cells were incubated on ice for 30 min with gentle mixing

every 10 min. To wash off unbound antibody 1.5 mL of ice-cold ASB was added to the cells followed by centrifugation at 400×g for 8 min, and aspiration of supernatant. The resulting cell pellets were disrupted and a dilution of the secondary antibody was added and staining/washing steps repeated as above. In the case of primary anti-HA and anti-FLAG antibodies 0.5 μg of anti-mouse IgG (H + L)-PE diluted in 50 μL of ASB were used and the primary anti-β2m pAb was followed up with 0.2 μg of goat anti-chicken-PE antibody or 0.2 μg anti-chicken-biotin. The anti-chicken-biotin was followed up with a dilution of 10 μL streptavidin-PE (Beckman Coulter) in 50 μL fluorescence activated cell sorting (FACS) buffer with washes between stains. Cells (10,000 per sample) were then analyzed for surface staining on the fluorescence (FL)-2 gate using a Cell Lab Quanta SC flow cytometer; Beckman Coulter.

3.12 Immunoprecipitation and Western blotting

Transfected HEK 293T cells were incubated for 48 h and then washed and detached as described above, and aliquoted (3×10^6) into 1.5mL Eppendorf tubes. After a D-PBS wash as described above the cells were lysed for 10 min with 500 μL of ice-cold immunoprecipitation (IP) buffer (50mM Tris–HCl, 150mM NaCl, 1% TritonX-100, supplemented with complete mini EDTA-free protease inhibitor tablets; Roche). Following centrifugation for 15 min at 16,000×g to remove cellular debris the lysates were transferred to a new 1.5 mL Eppendorf tube and incubated with 2 μg of either anti-HA mAb clone HA.C5, or anti-FLAG M2 mAb for 14–16 h at 4℃ on a rotary mixer. Protein G sepharose beads (GE Healthcare) were pre-washed with IP buffer and then 50 μL of beads were added to the samples and incubated for 2 h at 4℃. The beads were washed three times with 1 mL of IP buffer. Then 150 μL of 2×SDS-PAGE reducing buffer was added to the samples followed by boiling for 10 min and storage at −20℃, if required, prior to analysis. Twenty-five microlitre samples (equivalent to 0.5×10^6 lysed cells) were

electrophoresed on 10% SDS-PAG, transferred to 0.2 μm nitrocellulose membranes (BioRad) and then stained with Ponceau S (Sigma–Aldrich) to ensure successful transfer and equivalent loading of samples. The blots underwent blocking by incubation in Tris-buffered saline (TBS) supplemented with 0.1% Tween 20 (TTBS) and 5% skim milk (TTBS-SKIM) for 30 min at room temperature and were then incubated 14–16 h at 4℃ with anti-HA-HRP or anti-FLAG-HRP in TTBS-SKIM (1:1000, v/v). After two washes in TTBS and two washes in TBS the immunoreactive bands were detected using the SuperSignal West Pico Chemiluminescent Substrate kit (Pierce Biotechnology) followed by exposure to X-ray film (Kodak). In order to strip the membranes of bound antibody and re-probe with another antibody, blots were incubated for 10 min at room temperature in 100mL Western blot stripping buffer (15% glycine, 1% SDS, 0.01% Tween 20, pH 2.2) followed by two 10 min washes with 100 mL TBS, two 5 min washes with 100 mL of TTBS. Finally the membrane was incubated with TTBS-SKIM for 30 min prior to incubation with antibody.

Western blotting of proteins from lysates or culture supernatants of transfected HEK 293T cells followed the SDS-PAG and blotting procedures described for immunoprecipitates. For the analysis of proteins in the cell culture supernatant 2 μL of cell culture supernatant were added to 98 μL reducing buffer, while 50 μL of lysate was added to 50 μL of reducing buffer before boiling. Antibodies used for the detection of proteins on blots included HRP conjugated antibodies as listed in 3.3. The anti-HA and anti-FLAG antibodies were used as for the detection of proteins in blots of immunoprecipitates, the goat anti-mouse IgG3-HRP (Beckman Coulter) and anti-mouse IgG Fc antibody (US Biological) were used at a 1/1000 dilution in skim milk blocking buffer, and the goat anti-mouse IgG(H+L)-HRP (BioRad) was used at a 1/500 dilution in skim milk blocking buffer. Washes were done as described for blots of immunoprecipitates. The anti-mouse IgG, Fc antibody was followed up with a secondary anti-rabbit IgG(H+L)-HRP antibody at a 1/5000 dilution in skim milk blocking buffer. The anti-channel catfish β2m pAb was diluted 1/2500 in skim milk blocking buffer

before addition to the membrane. The membrane was washed as described above and a secondary anti-chicken-HRP antibody was added to the membrane in a 1/5000 dilution in skim milk blocking buffer. In the case of the AP conjugated antibody 2% BSA with 0.05% sodium azide in TTBS was used as a blocking agent instead of skim milk powder. The antibody incubation period was 14-16 h at 4℃ and the antibody was used at a 1/1000 dilution in blocking buffer. The blots were then washed as described for immunoprecipitations. For the development of the blots the AP development solution was mixed freshly prior to addition to the membrane: 10 µL BCIP solution (Roche), 10 µL NBT solution (Roche) per 10 mL of AP buffer (0.1M Tris HCl, pH 9.5; 0.1M NaCl; 0.05M MgCl2). The mixture was added onto the membrane and when satisfying colour development was reached the reaction was stopped by a gentle rinse with deionized water.

3.13 Coating of fluorescent protein G sephrose beads with IpLITR-Fc fusion proteins

Non-transfected HEK 293T cells served as controls and were grown in parallel with the transfected cells (see 3.12). Fourty eight h post-transfection all cells were washed 2 times with D-PBD/ETDA as described above and lysed in ice cold SephG binding buffer (20 mM NaPO4, pH 7 with protease inhibitor tablets (Roche)) by pulling them through a 18G syringe several times followed by three freeze-thaw cycles of 20 min at -80℃ and 20 min at 37℃. Following centrifugation for 15 min at 16,000×g to remove cellular debris the lysates were transferred to a new 1.5 mL Eppendorf tube. The lysates were stored at -20℃. Fifty µL of Fluoresbrite™ YG protein G sepharose 1.0 micron microspheres (Polysciences) were washed three times with 1 mL of PBS supplemented with 10 mg/mL BSA. To coat the beads with Fc-fusion proteins or with control lysates 500 µL of lysate diluted in 500 µL of BSA supplemented PBS were added to the washed beads and then incubated at 4℃ for 14 h with rotational mixing.

Following incubation the beads were centrifuged for 5 min at 10,000 rpm at 4℃. The supernatant was removed and stored at 4℃ for future analysis. The beads were washed three times as above. Finally, the beads were resuspended in 500 µL PBS/BSA and stored in the dark at 4℃. To verify coating of the protein G beads with Fc-fusion proteins 10 µL of the beads or the supernatant were reduced in an equal volume of reducing buffer as described above (see 3.12) and separated on an 10% SDS-PAG. The proteins were transferred onto a nitrocellulose membrane and blotted with the anti-HA antibody as described in 3.12.

3.14 Incubation of catfish B cells with IpLITR-Fc fusion proteins coated protein G sepharose beads

The catfish B cell lines 3B11 and 1G8 were used target cells to test staining with coated protein G beads. Two hundred fifty thousand cells were transferred to a 1.5 mL Eppendorf tube and for each stain 120 µL of various dilutions (PBS/BSA) of bead solutions were added (1/1, 1/5, 1/10, 1/50, 1/100). The cell-bead mixture was incubated at the catfish cell physiological temperature of 27℃ and 5% CO_2 for 30 min with gentle mixing every 10 min. The cells were then washed as described above (see 3.12) and resuspended in 120 µL of PBS/BSA. Cells (10,000 per sample) were then analyzed for surface staining on the FL-2 gate using a Cell Lab Quanta SC flow cytometer; Beckman Coulter.

3.15 Staining of catfish B cells with crude IpLITR-Fc fusion proteins

HEK 293T cells were transfected, incubated and lysed as described above (see 3.10 and 3.12). Following centrifugation for 15 min at 16,000×g to remove cellular debris the lysates were transferred to a new 1.5mL Eppendorf tube. The crude lysates were stored at -20℃ until ready for use. Two hundred fifty thousand 3B11 cells were used in each stain with 1/1, 1/10 or 1/100 dilutions of lysate. The cells were incubated and washed as above (see 3.12) followed by addition of 2 µg

anti-IgG3-PE antibody or 2 μg anti-HA primary antibody. The incubations and washes were as described above. In the case of the anti-HA antibody the staining was followed up with a secondary anti-mouse-PE antibody in the same manner as described before. Cells (10,000 per sample) were then analyzed for surface staining on the FL-2 gate using a Cell Lab Quanta SC flow cytometer; Beckman Coulter.

Table 3.1. List of primers used in this thesis[a].

Primer name	Primer sequence 5' to 3'
pDisplay_IpLITR_SmaI_Fwd	CCCGGGGTTCTGTCTGTGGAGCCG
pDisplay_IpLITR_SalI_Rvs	GTCGACTCATCAGACAGACGCTTCAGCTTCTAT
pCMV-9_IpFcRγ_HindIII_Fwd	AAGCTTGAGGCAAACGTC TGCTATA
pCMV-9_IpFcRγ_BamHI_Rvs	GGATCCTTATGATTTGGTCTTCAAGTT
pCMV-9_IpFcRγ-L_HindIII_Fwd	AAGCTTGAGGGTGGATTGTGTTATA
pCMV-9_IpFcRγ-L_BamHI_Rvs	GGATCCTTATGCCAAAGGTTCCTTCT
pCMV-9_IpCD3ζ-L_HindIII_Fwd	AAGCTTCTACAGCCCAATGACCCCA
pCMV-9_IpCD3ζ-L_BamHI_Rvs	GGATCCTCAGTCGCGTGATGGTGGTA
pCMV-9_IpDAP12_HindIII_Fwd	GCGGCCGCGCAAATCAAGACTGCAGTTCCTG
pCMV-9_IpDAP12_BamHI_Rvs	GGATCCTTATTTCCGATACTGCTGAAG
pCMV-9_IpDAP10_HindIII_Fwd	AAGCTTGATGGAGACAAGTACTCATG
pCMV-9_IpDAP10_BamHI_Rvs	GGATCCTCATGTTTTGCAGTTTGC
pCMV-9_DrFcRγ_HindIII_Fwd	AAGCTTGCTGATGCGATGAGTCTG
pCMV-9_DrFcRγ_BamHI_Rvs	GGATCCTCATTTTTTGGCGGATTTCAT
pCMV-9_DrFcRγ-L_HindIII_Fwd	AAGCTTCAACAGAGTGAAAATGTGTGT
pCMV-9_DrFcRγ-L_XbaI_Rvs	TCTAGATTAAGCGAGAGGTTTTTTCT
pCMV-9_DrCD3ζ_HindIII_Fwd	AAGCTTACCTCCTTCTATGACCCCACA
pCMV-9_DrCD3ζ_BamHI_Rvs	GGATCCTTAGCGCGGTGGCAACGG
pCMV-9_DrCD3ζ-L_HindIII_Fwd	AAGCTTACTGTAAGCGACCCGACTT
pCMV-9_DrCD3ζ-L_BamHI_Rvs	GGATCCTCATACAGTAGGAAACGTTTCC
pCMV-9_DrDAP12_NotI_Fwd	GCGGCCGCAAATCAAGACTGCAGTTCCTG
pCMV-9_DrDAP12_BamHI_Rvs	GGATCCTTATTTCCGATACTGCTGAAG
pCMV-9_DrDAP10_HindIII_Fwd	AAGCTTGATGGAGACAAGTACTCATG
pCMV-9_DrDAP10_BamHI_Rvs	GGATCC TCA TGT TTT GCA GTT TGC
pFuse_IpLITR_EcoRV_Fwd	GATATCAGTTCTGTCTGTGGAGCC
pFuse_IpLITR_BglII_Rvs	AGATCTAGTGAGTGTAACAGTGTTGCTGG
pFuse_IpLITR_NcoI_Rvs	CCATGGCAGTGAGTGTAACAGTGTTGCT
pFuse-pDisplay_IpLITR_SalI_Rvs	GTCGACTCATCATTTACCAGGGGAGCGAGACAGGTT
pFuse-BWZ_XhoI_Fwd	CTCGAGATGTACAGGATGCAACTCCTGTCTTGC
pFuse-BWZ_NotI_Rvs	GCGGCCGCTTTACCAGGGGAGCGAGACAGGTT
pFuse-pDisplay-BWZ_XhoI_Fwd	CTCGAGATGGAGACAGACACACTCCTGCTATG
D4_mFc_pFuse_BWZ_Fwd	AAATGTCCAAGAAGAAGGTTAGTCTG
pFuse-pDisplay_BWZ_internal_Fwd	AGTGTGGAGTGGGAAAGGAA
BWZ_IpLITR_2.6b_internal_Rvs	CGCTGACCCTCCATCAATAG
BWZ_IpLITR_1-3_internal_Rvs	CGTTGTCCGTCCATAAATAG
pDisplay_IpMHCIalpha_SmaI_Fwd	CCCGGGGTCACACACTCTCTACAGTA
pDisplay_IpMHCIalpha_SalI_Rvs	GTCGACTTAAGAGTTGTTGGAGGAAGAATC
pCMV-9_IpMHCIalpha_NotI_Fwd	GCGGCGCGGTCACACACTCTCTACA
pCMV-9_IpMHCIalpha_EcoRV_Rvs	GATATCTTAAGAGTTGTTGGAGGAAGAATC
T7_Fwd	TAATACGACTCACTATAGGG
BGH_Rvs	TAGAAGGCACAGTCGAGG
pCMV-9_Ipb2m_HindIII_Fwd	AAGCTTAAGGAGTCTCCACCAAAAATC
pCMV-9_Ipb2m_BamHI_Rvs	GGATCCTTACATGTCTGGCTCCCAG
pFuse_Ipb2m_EcoRV_Fwd	GATATCGAAGGAGTCTCCACCAAAATC
pFuse_Ipb2m_BglII_Rvs	AGATCTTTACATGTCTGGCTCCCAG

[a] All primers were obtained from Integrated DNA Technologies (IDT).

CHAPTER 4

IDENTIFICATION OF THE ITAM-CONTAINING SIGNALLING ADAPTOR MOLECULES RECRUITED BY STIMULATORY IpLITR-TYPES[1]

4.1 Introduction

Findings from studies of innate immune receptors in non-mammalian vertebrates indicated that certain signalling pathways mediated by these receptors are conserved among vertebrates. The characterization of such pathways provides important information regarding the evolution and function of immunoregulatory networks in vertebrates. A diverse array of innate immune receptors in birds [25, 181], frogs [26, 134, 182] and fish [183, 184] have the ability to recruit inhibitory phosphatases or assemble with ITAM-containing adaptors. In support of these findings, recent work reinforced that conserved inhibitory signalling mechanisms contribute to IpLITR-mediated control and coordination of immune cell effector functions [168].

IpLITRs are believed to control multiple facets of cellular immunity through the induction of both inhibitory and stimulatory signals [28]. The study presented here focussed on the biochemical characterization of putative stimulatory IpLITR-types that contain a charged TM region due to the presence of a single lysine residue [28]. I hypothesized that these proteins specifically assemble with teleost ITAM-containing adaptor proteins as a requirement for receptor surface expression. Teleost ITAM-containing signalling adaptor proteins share the basic structural features of mammalian signalling adaptors that associate with a wide variety of immune receptors [184]. Signalling adaptors FcRγ chain, FcRγ-like molecule (FcRγ-L), CD3ζ, CD3ζ-like molecule (CD3ζ-L), DAP10 and DAP12 have been identified in zebrafish [184] and the channel catfish [185]. These signalling adaptors contain ITAM motifs or, in the case of DAP10, an

[1] A version of this chapter has been published. Mewes *et al.*, 2009. Mol Immunol. **47**(2-3): p. 318-31.

YxxM motif (Fig 4.1), analogous to mammalian signalling adaptors. The presence of FcRγ-L and CD3ζ-L molecules in teleost is most likely due to a gene duplication event [185]. Pairwise amino acid comparison between catfish and zebrafish signalling adaptor homologues revealed that these molecules have approximately 50% sequence identity [185].

To address the hypothesis that IpLITRs associate with teleost signalling adaptors, I used cationic polymer-mediated transient transfections of HEK 293T cells in conjunction with flow cytometry and co-immunoprecipitations. In this chapter, I report that IpLITR 2.6b, as a representative of putative stimulatory IpLITR-types, associated with the channel catfish and the zebrafish FcRγ homologues and CD3ζ homologues. I also report that only the association with teleost FcRγ or FcRγ-L results in high levels of surface expression of IpLITR 2.6b.

4.2 Results

4.2.1 Expression of catfish ITAM-containing signalling adaptor molecules in a eukaryotic cell line

The teleost signalling adaptor molecules were cloned into an expression vector that adds a triple (3x) FLAG tag N-terminal to the adaptor as well as a mammalian preprotrypsin (PPT) leader peptide to facilitate surface expression (Fig. 4.2). HEK 293T cells were transiently transfected with individual ITAM-containing adaptor constructs and the adaptor proteins were detected on the cell surface by flow cytometry following anti-FLAG epitope staining (Fig. 4.3). When compared to mock-transfected cells, cell staining increased by ~87% for IpFcRγ, ~84% for IpFcRγ-L, ~86% for IpCD3ζ-L, ~79% for IpDAP12 (Fig 4.3) and ~60% for IpDAP10. The surface expression of zebrafish signalling adaptors when compared to mock transfections was: DrFcRγ ~83%, DrFcRγ-L ~82%, DrCD3ζ-L ~64%, DrCD3ζ-L ~47%, DrDAP12 ~75% and DrDAP10 ~77%. Whole cell

lysates of transfected HEK 293T cells were also probed with an anti-FLAG antibody after transferring the proteins onto nitrocellulose membranes. Proteins of the expected sizes for each adaptor were readily detected in the lysates of transfected cells for IpFcRγ (~18 kDa), IpFcRγ-L (~18 kDa), IpCD3ζ-L (~22 kDa), and IpDAP12 (~20 kDa) (Fig. 4.4). IpDAP10 was also expressed on the surface of transfected HEK 293T cells and detected by Western blotting in the lysates [185]. The sequence for IpCD3ζ has recently been deposited in GenBank (FJ809774.1) but was not available at the time of these studies.

4.2.2 Examination of the association between IpLITR 2.6b and channel catfish ITAM-containing adaptors

IpLITR 2.6b was cloned into an expression construct which adds an HA-epitope tag N-terminal to the receptor as well as a mammalian Ig κ leader peptide to facilitate surface expression in mammalian cells (Fig. 4.5). To examine whether IpLITR 2.6b assembles with a teleost ITAM-containing signalling adaptor, I performed co-immunoprecipitations from lysates of transfected HEK 293T cells (see scheme outlined in Table 4.1). After immunoprecipitation with an anti-HA antibody, an HA-IpLITR 2.6b (~27 kDa) immunoreactive band was observed in all samples of transfected cells when probed with an anti-HA-HRP antibody on a Western blot (Fig. 4.6). However, the HA-IpLITR 2.6b band was absent in mock-transfected cells (Fig. 4.6). The bands observed on the Western blots after anti-HA immunoprecipitations were more pronounced from samples derived from HEK 293T cells that were co-transfected with HA-IpLITR 2.6b and IpFcRγ-L and/or IpCD3ζ-L, in contrast to the fainter bands observed in the IpDAP12 co-transfected sample and the single transfected HA-IpLITR 2.6b sample.

Western blots were also performed from the co-immunoprecipitated HEK 293T lysates (i.e. cells transfected with IpLITR and adaptor combinations) after they were stripped and re-probed with an anti-FLAG-HRP antibody, to examine an association between IpLITR 2.6b and the signalling adaptors. Anti-FLAG

reactive proteins (i.e. catfish adaptors) were not detected in the immunoprecipitates from mock-treated samples, from single transfected cells with HA-IpLITR 2.6b alone or from the immunoprecipitates obtained from HEK 293T cells co-transfected with HA-IpLITR 2.6b and IpDAP12. However, in the immunoprecipitates from HEK 293T cells co-transfected with HA-IpLITR 2.6b and either IpFcRγ-L and/or CD3ζ-L bands could be observed for IpFcRγ-L (~18 kDa) and CD3ζ-L (~22 kDa) (Fig. 4.6). IpFcRγ also associates with IpLITR 2.6b [185]. Cells co-transfected with HA-IpLITR 2.6b and IpFcRγ-L and IpCD3ζ-L showed association of both signalling adaptors to the catfish receptor, whereas co-transfections of the receptor with combinations of IpFcRγ-L and IpDAP12 as well as IpCD3ζ and IpDAP12 only resulted in detectable co-immunoprecipitation of IpFcRγ-L (~18 kDa) or IpCD3ζ-L (~22 kDa) but not IpDAP12 (~20 kDa) to IpLITR 2.6b under the tested conditions (Fig. 4.6).

4.2.3 Augmented surface expression of IpLITR 2.6b by associations with teleost FcRγ and FcRγ-L ITAM-containing adaptors

HEK 293T cells were transfected with an HA-IpLITR 2.6b construct containing the Ig κ leader sequence. These cells show an increase in HA epitope staining of ~10% in comparison to mock-transfected cells when analyzed via flow cytometry (Fig. 4.7). Cells co-transfected with this HA-IpLITR 2.6b construct in conjunction with ITAM-containing adaptors IpFcRγ or IpFcRγ-L displayed a consistent and reproducible increase in HA epitope staining of ~30% when compared to cells transfected with HA-IpLITR 2.6b alone (Fig. 4.7). Co-transfection of HA-IpLITR 2.6b with IpCD3ζ-L or IpDAP12 did not result in increased surface expression of the receptor reflected by minimal HA staining (Fig. 4.7).

Moreover, HA-IpLITR 2.6b transfected HEK 293T cells demonstrated a >30% increase in HA epitope staining when co-transfected with DrFcRγ or DrFcRγ-L in comparison to cells transfected with HA-IpLITR 2.6b alone (Fig.

4.8). On the contrary, HEK 293T cells co-transfected with IpLITR 2.6b and the zebrafish adaptors CD3ζ, CD3ζ-L or DAP12 did not result in increased surface staining, confirming that these teleost signalling adaptors do not enhance surface expression of IpLITR 2.6b (Fig. 4.8). Neither zebrafish DAP10 nor channel catfish DAP10 augmented the surface expression of IpLITR 2.6b [185].

4.3 Discussion and conclusions

To further study the role of IpLITRs as potential players in the regulation of cellular immunity in teleost, I characterized stimulatory IpLITR-types and examined their signalling potential. Since there are no monoclonal antibodies available against any IpLITR types or teleost signalling adaptors at this time, my studies relied on the expression of epitope tagged constructs in HEK 293T cells. Even though this approach is widely used in comparative immunology [25, 26, 168], there are obvious intrinsic limitations. Results obtained from these studies need to be verified in their natural system, which in the case of IpLITRs are catfish immune cells. Studies would ideally have to be performed without epitope tags using monoclonal antibodies. Therefore, results obtained with the current methods regarding IpLITR-adaptor associations need to be interpreted with caution. However, my initial results allow novel biochemical insights into the properties of stimulatory IpLITR-types, verifying that they have the potential to associate with teleost ITAM-containing signalling adaptors. Specifically, my results suggest that IpLITR 2.6b may preferentially associate with the teleost adaptors FcRγ chain and FcRγ-L chain as a requirement for increased cell surface expression. That IpLITR 2.6b could only be expressed at high levels on the cell surface with an appropriate adaptor was expected, since stimulatory receptor types are intracellularly retained when not associated with an appropriate adaptor as outlined in Table 4.1. [186]. This suggests that IpLITR 2.6b and the FcRγ/ FcRγ–L chain adaptor complexes co-localize when expressed on the cell surface, which could be verified in future studies using confocal microscopy.

The observation that transfection of IpLITR 2.6b alone resulted in increased surface staining (10-15%) when compared to mock-transfected HEK 293T cells was consistent and may be a result of over-expression in a human cell line. Such a phenomenon has been described in other studies as well [25, 26]. Another possibility for this trend is that IpLITR 2.6b may be expressed at low levels on the cell surface *in vivo*, independent of an association with an adaptor protein. However, the significantly increased surface staining in cells co-transfected with the FcRγ and FcRγ-L adaptors suggests that these may be the functionally significant adaptor molecules that are required to be associated with the receptor to promote its surface expression [186]. The co-immunoprecipitation experiments performed in this study suggest that IpLITR 2.6b can also assemble with CD3ζ and CD3ζ-L molecules, but the flow cytometric results indicated that the co-transfection of these adaptors with IpLITR 2.6b did not lead to increased receptor surface expression in HEK 293T cells.

The discovery that IpLITR can be co-immunoprecipitated with FcRγ chain homologues and CD3ζ homologues from transfected HEK 293T cells was surprising. I expected that, since only the FcRγ chain homologues increase the surface expression of the receptor, only FcRγ chain homologues can associate with IpLITR 2.6b. The immunoprecipitation experiments only showed bands in co-transfections with FcRγ chain and CD3ζ homologues. However, the possibility that IpLITR 2.6b associates with other signalling adaptors cannot be entirely excluded. Reduced levels of IpLITR 2.6b protein in co-transfection experiments with DAP12 in comparison to co-transfections with FcRγ chain and CD3ζ homologues are suggested by a consistent trend in reduced HA band intensity in all independent experiments. This brings with it limitations in sensitivity, should there be a so far undetected association between the receptor and DAP12. The results could alternatively suggest that reduced protein stability is the result of a lack of a stabilizing interaction with an adaptor. The reduced receptor band intensity could also be a result of an independent unknown effect, and in order to exclude the possibility that IpLITR 2.6b can promiscuously associate with any

teleost signalling adaptor intracellularly in HEK 293T cells, several experiments could be performed. To improve sensitivity lysates from DAP12 and IpLITR 2.6b co-transfected cells could be pooled prior to immunoprecipitation to counteract for lower IpLITR levels in a given sample and determine if this poses a detection limit for the association with an adaptor molecule. Another way to study an association between IpLITRs and signalling adaptors would be to use fluorescence resonance energy transfer (FRET). The receptor and the signalling adaptor could be labelled with the appropriate fluorescence tags and the presence of fluorescence could be determined using fluorescence microscopy. The results from co-immunoprecipitation studies following triple transfection experiments with IpLITR 2.6b and channel catfish adaptors FcRγ-L and CD3ζ also opened the possibility that catfish FcRγ-L and CD3ζ-L can form heterodimeric complexes. Similarly, the substitution of the CD3ζ subunit for FcRγ in FcγRI expression and function has been described in humans [187]. Moreover, the human FcRγ chain has been shown to be able to replace the CD3ζ chain in TCR signalling by CD4-positive T cells [188]. Functional FcRγ-CD3ζ heterodimers have also been suggested to mediate Fc receptor signal transduction in humans [189]. Since IpLITRs are expressed in a variety of leukocytes [28], it is possible that they can signal through the CD3ζ or FcRγ homologues, depending on the type of immune cell in which they are expressed.

The association between the IpLITR 2.6b and channel catfish signalling adaptors is likely based on conserved motifs or charges in their TM [185]. The presence of a lysine in the TM of mammalian activating receptors may be associated with the recruitment of DAP12 to the receptor TM [46]. Interestingly, IpLITR 2.6b encodes a lysine in its TM and recruits FcRγ chain orthologs. The presence of an arginine residue in the receptor TM in mammals promotes FcRγ chain orthologues recruitment via the FcRγ TM encoded aspartic acid [46]. For Zebrafish novel immune-type receptor (NITR) 9, this principle does not appear to hold true. This receptor contains an arginine residue in its TM, and this residue

together with the aspartic acid within the adaptor TM are required for the recruitment of DAP12 [183]. Therefore, it seems that, while in mammals there is a trend regarding the recruitment of signalling adaptors FcRγ to an arginine and DAP12 to a lysine the receptor TM [46], in fish, the recruitment of a signalling adaptor to the TM of an immune receptor cannot be predicted by what is known from mammalian receptors.

These initial studies indicate that stimulatory IpLITR-types do indeed have a signalling potential. They recruit ITAM bearing signalling adaptors. However, the HEK 293T cell line, in which the receptor-adaptor associations were studied, are somewhat ill-fitted for studies of immune cell signalling, since these cells lack any immune cell functions by default. Therefore, future studies will be carried out in transfected immune cells that contain all the components and signalling pathways required to activate the cell following receptor cross-linking or ligand binding. The activation of the immune cell can then be detected and quantified with assays that measure processes like phagocytosis and cytokine release.

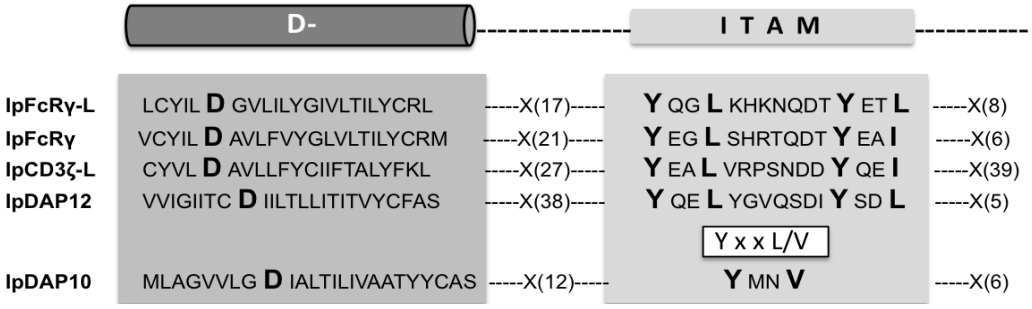


Figure 4.1. Channel catfish signalling adaptors contain a lysine residue in their TM and an ITAM or YxxM motif in their cytoplasmic tail. Predicted TM segments and signalling motifs were aligned using Clustal W.

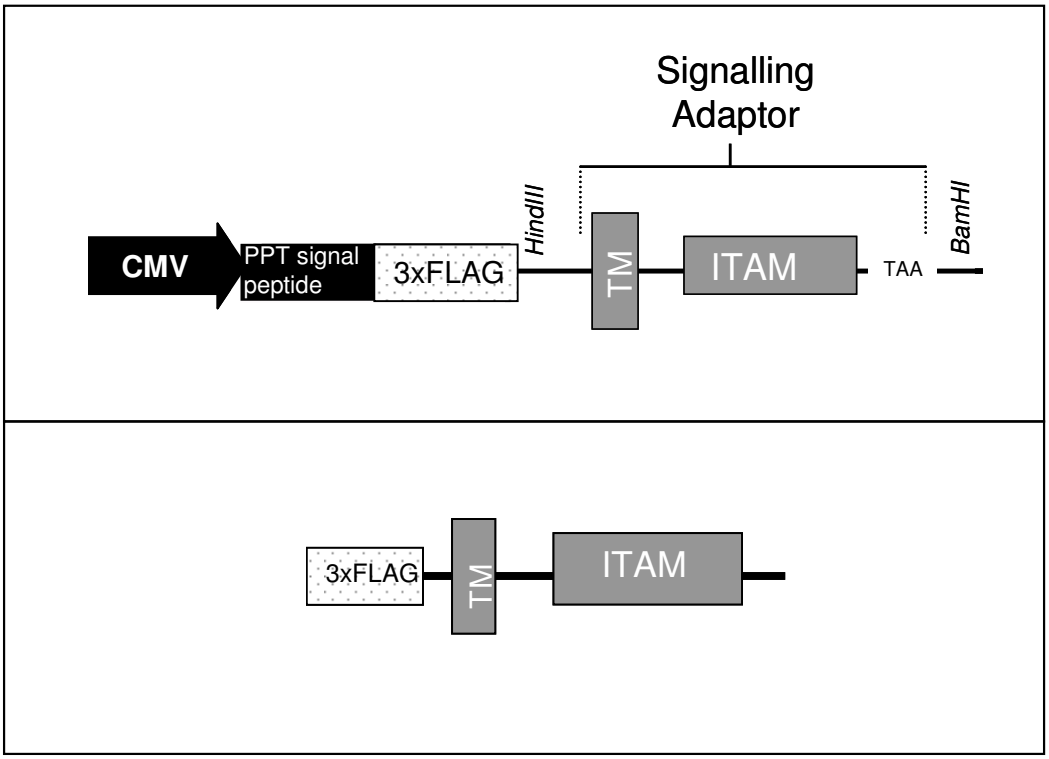


Figure 4.2. Teleost signalling adaptor constructs for surface expression and detection in HEK 293T cells. Signalling adaptor constructs were cloned into the p3XFLAG-CMV™-9 vector using HindIII and BamHI restriction sites. The vector contains a CMV promoter to drive expression of the cloned gene. The resulting construct has an N-terminal triple FLAG epitope tag and the mammalian preprotrypsin (PPT) leader signal allows for surface expression of this recombinant catfish protein in a mammalian cell line.

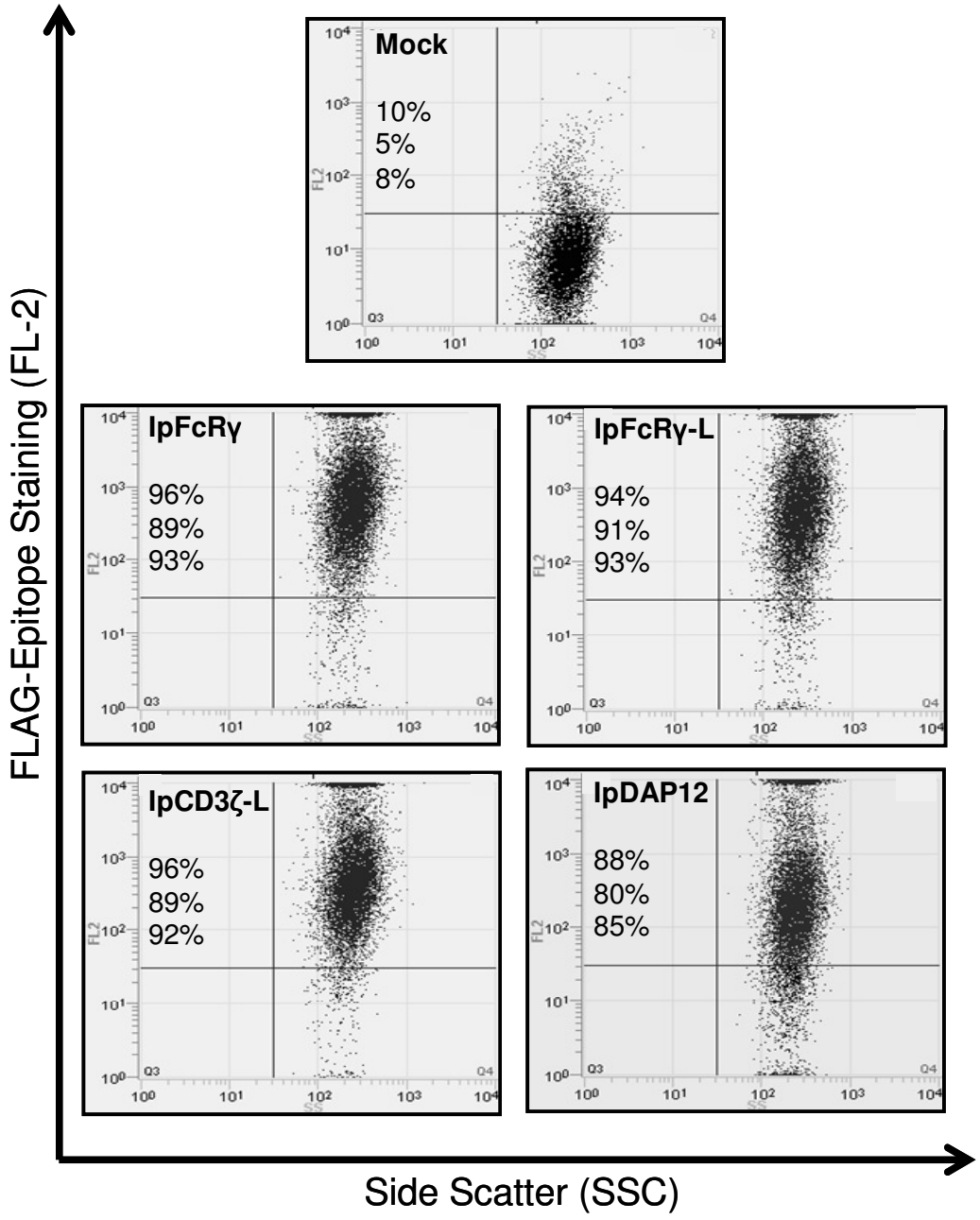


Figure 4.3. Surface expression of catfish signalling adaptor constructs in HEK 293T cells. HEK 293T cells were transiently transfected with catfish signalling adaptor constructs containing FLAG epitope tags. Following 48 hours of incubation, the transfected cells were stained with 2 μg of mouse anti-FLAG antibody, washed and then incubated with an anti-mouse PE conjugated secondary antibody. The cells were the analyzed on a flow cytometer. Results for independent experiments are shown as (%) in each of the panels. The (%) in the top row of each panel corresponds to the representative data displayed.

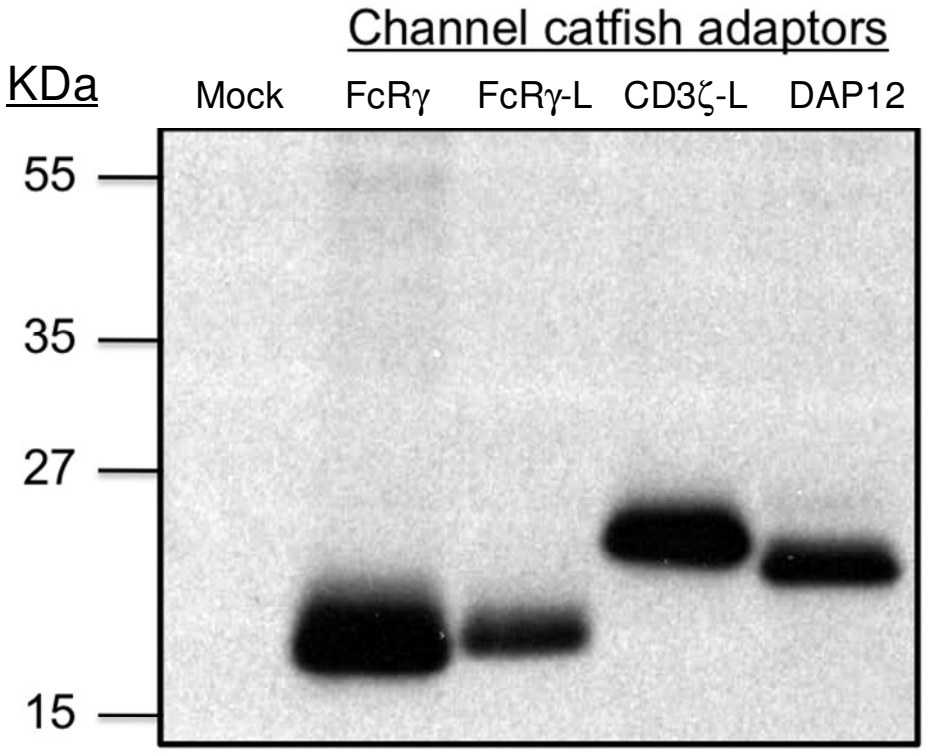


Figure 4.4. Western blot of HEK 293T whole cell lysates transfected with channel catfish signalling adaptor constructs. Twenty five microlitres of the lysates were separated on a 10% SDS gel under reducing conditions. The proteins were transferred onto a nitrocellulose membrane and detected with an anti-FLAG-HRP antibody. Results are representative of three independent experiments.

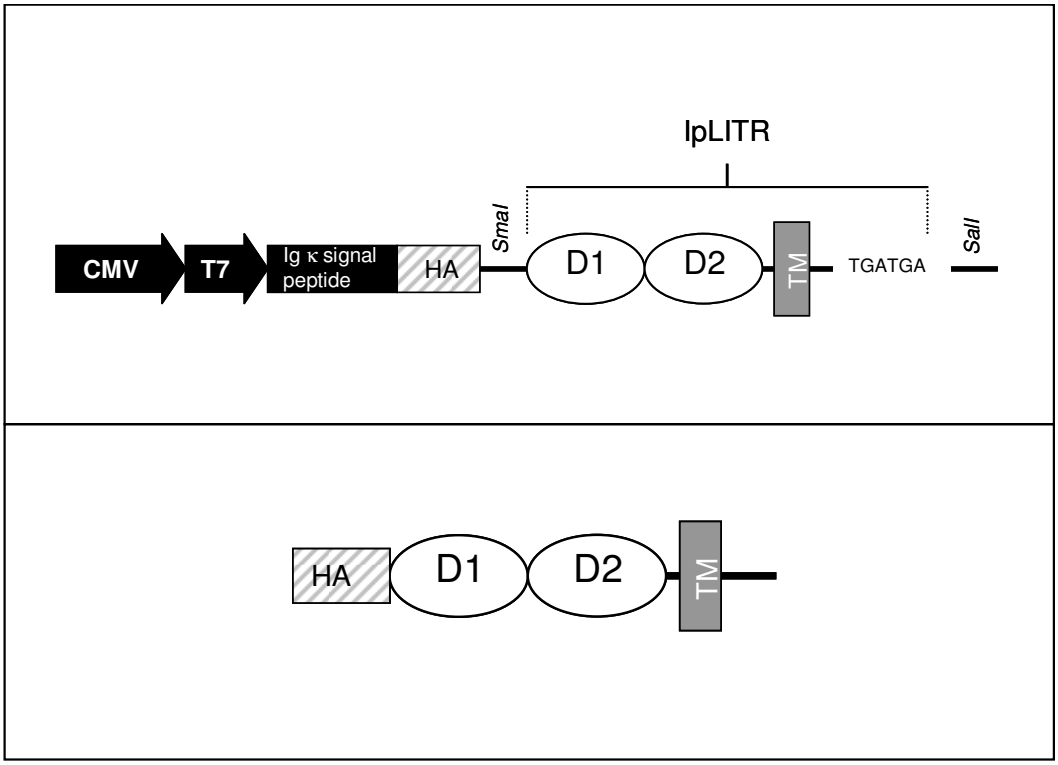
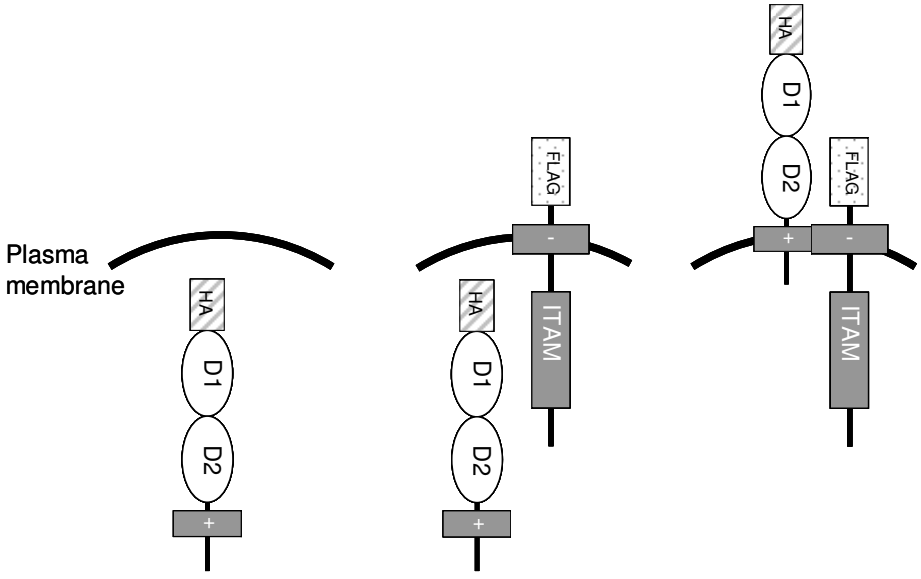


Figure 4.5. IpLITR 2.6b construct for surface expression and detection in HEK 293T cells. IpLITR constructs were cloned into the pDisplay™ vector using SmaI and SalI restriction sites. The vector contains a cytomegalovirus promoter (CMV) to drive transcription of the cloned recombinant gene. Additionally, the pDisplay™ vector encodes a mammalian TM following the Sal I site (TM not shown). Two stop codons terminate translation past the IpLITR C-terminus. The resulting construct has an N-terminal HA epitope tag and the mammalian Ig κ leader signal allows for surface expression of this recombinant catfish protein in a mammalian cell line.

Table 4.1. Experimental outline and expected results for receptor-adaptor association studies. LITR 2.6b receptor expression constructs contain an N-terminal HA epitope tag and teleost signalling adaptor expression constructs contain an N-terminal 3xFLAG epitope tag. The surface expression and immunoprecipitation experiments were performed in transfected HEK 293T cells.

Transfection:	**Receptor alone**	**Receptor and inappropriate adaptor**	**Receptor and appropriate adaptor**
Expected results:			
Surface staining and flow cytometry:	No receptor surface expression / no HA staining	No receptor surface expression / no HA staining	Receptor surface expression / HA staining
Immunoprecipitation of receptor and Western analysis:	Receptor (HA) band	Receptor (HA) band No association / no adaptor (FLAG) band	Receptor (HA) band Association / Adaptor (FLAG) band



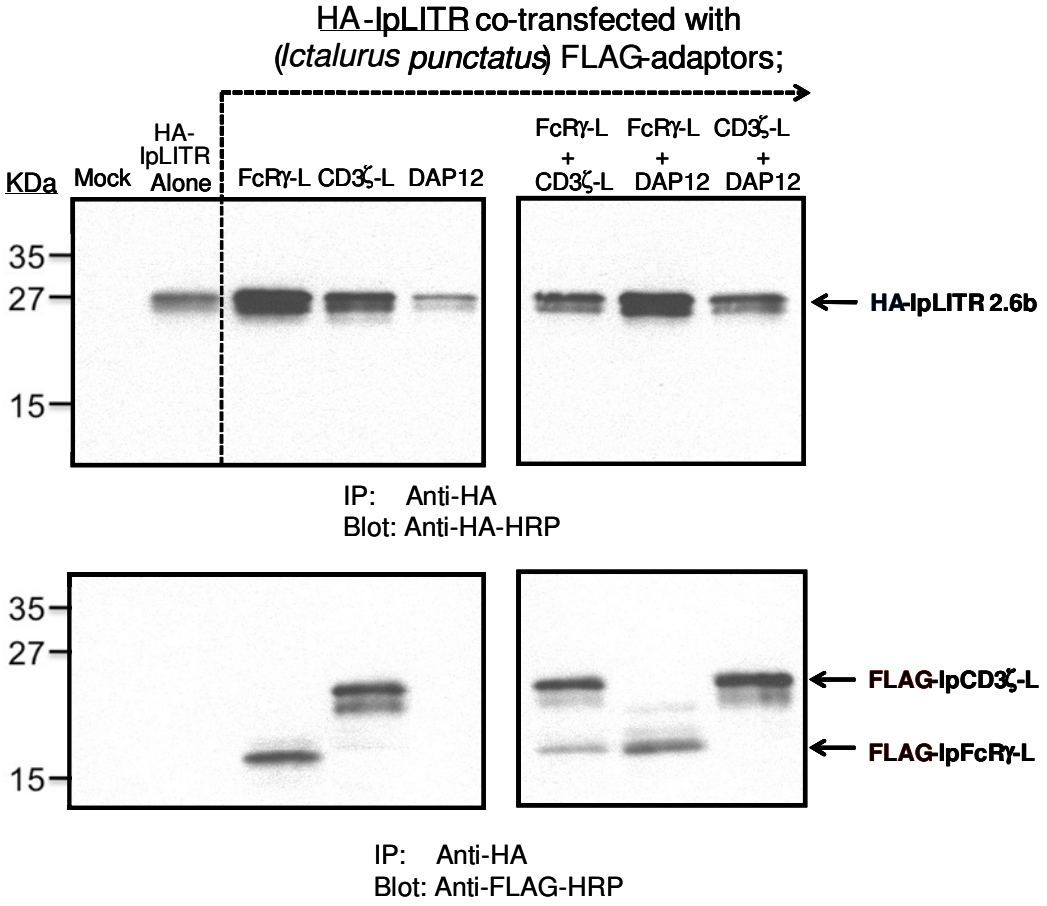


Figure 4.6. IpLITR 2.6b associates with channel catfish signalling adaptors FcRγ-L and CD3ζ-L chains but not with DAP12. Co–immunoprecipitation experiments were performed in HEK 293T cells transfected with epitope tagged constructs of N-terminal HA-tagged IpLITR 2.6b alone or in combinations with N-terminal FLAG-tagged adaptor constructs. Immunoprecipitations were performed using 2 µg of anti-HA antibody and the immunoprecipitates were separated on a 10% SDS-PAG under reducing conditions. Western blots were probed with either an anti-HA antibody to detect IpLITR 2.6b (upper blot) or anti-FLAG antibody to detect catfish signalling adaptors (bottom blot). Results are representative of three independent experiments.

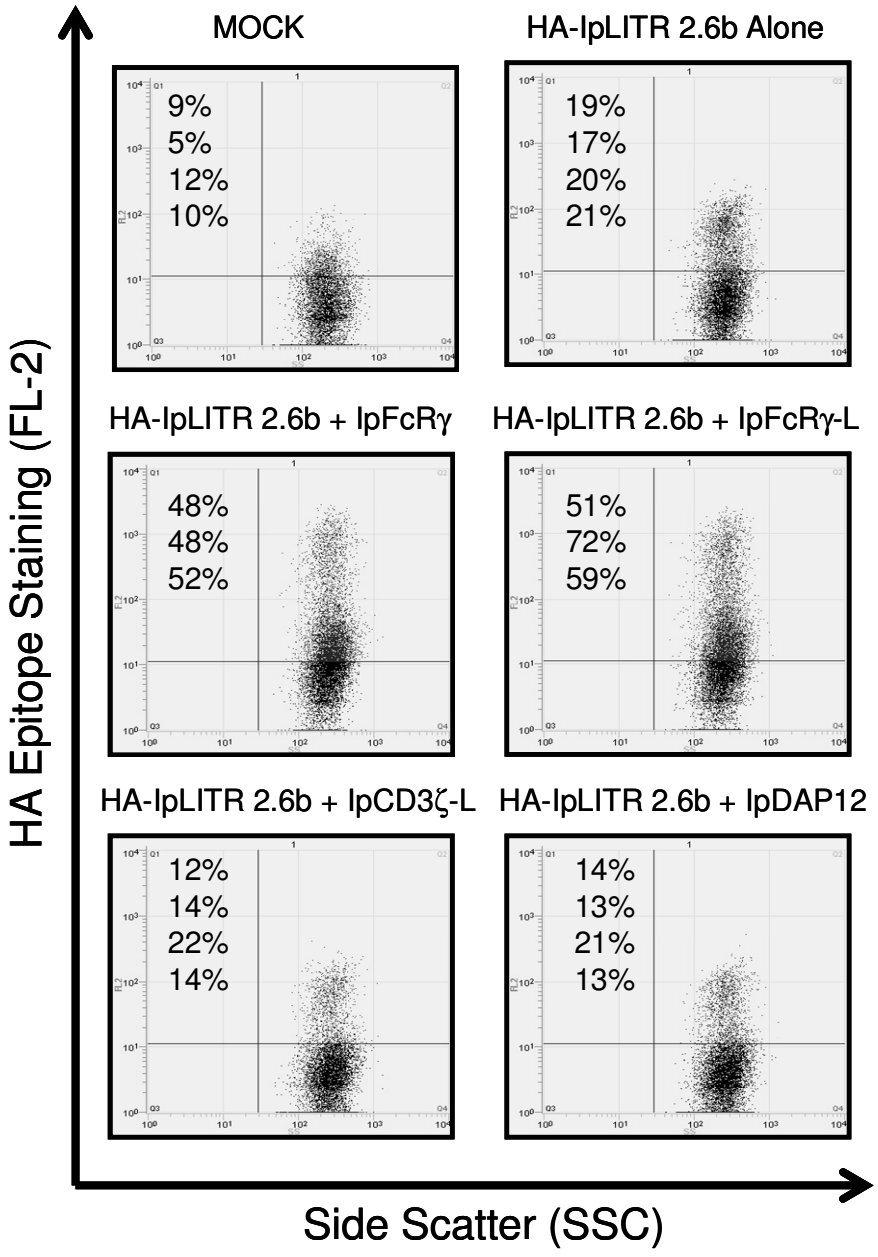


Figure 4.7. Surface expression of IpLITR 2.6b is augmented in the presence of catfish signalling adaptors FcRγ and FcRγ-L in HEK 293T cells. HEK 293T cells were transiently transfected with HA-IpLITR 2.6b and catfish signalling adaptor constructs. Following 48 hours of incubation, the transfected cells were stained with mouse anti-HA antibody, washed and incubated with anti-mouse-PE secondary antibody. The cells were then analyzed on a flow cytometer. Results for independent experiments are shown as (%) in the panels and the (%) in the top row of each panel correspond to the representative data displayed.

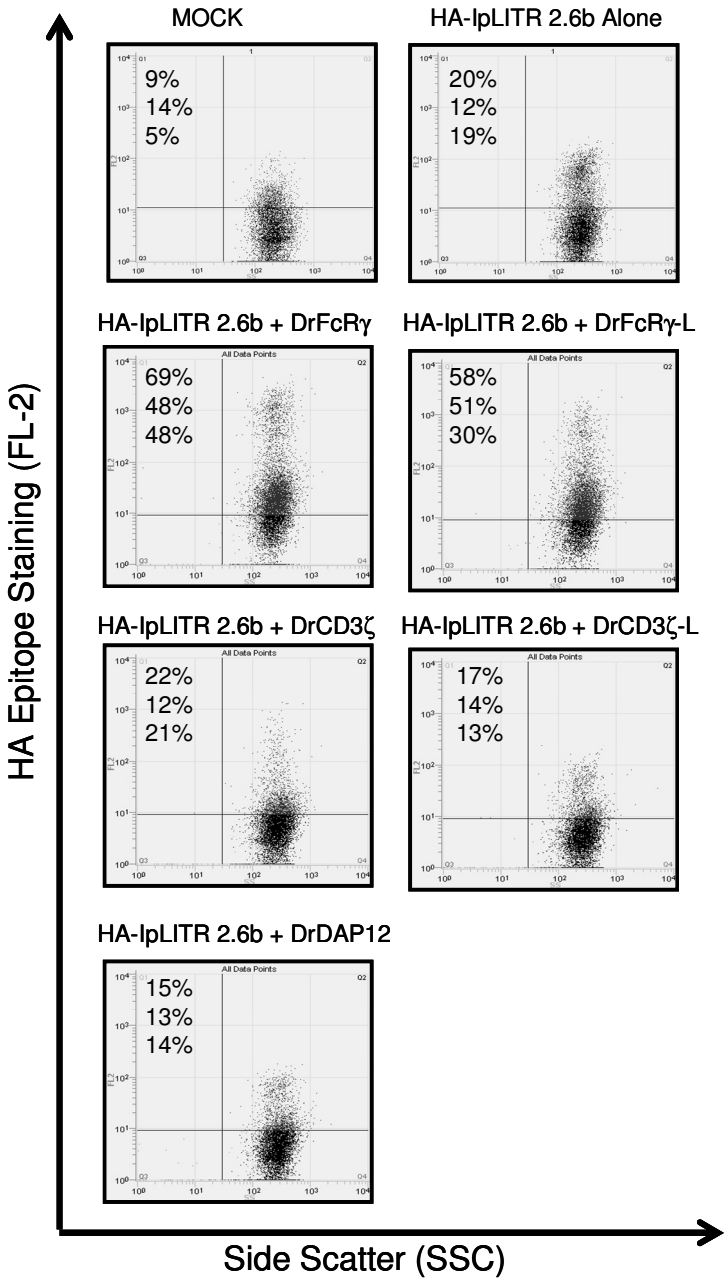


Figure 4.8. Surface expression of IpLITR 2.6b is augmented in the presence of zebrafish signalling adaptors FcRγ and FcRγ-L in HEK 293T cells. HEK 293T cells were transiently transfected with HA-IpLITR 2.6b and zebrafish signalling adaptor constructs. Following 48 hours of incubation, the transfected cells were stained with a mouse anti-HA antibody, washed and incubated with an anti-mouse-PE secondary antibody. The cells were then analyzed on a flow cytometer. Results for independent experiments are shown as (%) in the panels and the (%) in the top row of each panel correspond to the representative data displayed.

CHAPTER 5

DEVELOPMENT OF ASSAYS FOR THE DISCOVERY OF IpLITR BINDING PARTNERS

5.1 Introduction

The identification of immune receptor ligands is essential to fully understand and appreciate the contribution of specific immune receptor proteins to the function of immune cells in which they are naturally expressed. This is not only relevant for novel discoveries like NITRs and CHIRs but also for long known receptor proteins. Despite the fact that the NK receptor P1 (NKR-P1) was discovered in the late 1970s [190], a ligand for this receptors had not been identified until 2004 [191].

The latest strategies used for the discovery of immune receptor ligands or identification of cells that express them on their surface, all use common approaches. Such techniques include the generation of soluble receptor constructs as staining agents alongside cell based reporter assays as tools to screen for receptor ligands [192].

A popular strategy for the generation of soluble fusion proteins is the generation of chimeric Fc-fusion proteins. The extracellular receptor domains presumed to be involved in ligand recognition are fused to human IgG1 Fc domains (Cγ2 and Cγ3). Such fusion proteins have been constructed for NITRs [193], TREMs [20, 21] and CHIRs [192]. HEK 293T cells are relatively easy to transfect with commonly available cationic polymer transfection reagents. They are therefore a popular choice to transiently express proteins. For this reason, the receptor constructs expressed in HEK 293T cells have an added mammalian secretion signal. Antibodies are not available for many of the recently discovered immune receptors. This is why epitope tags are often introduced into the recombinant fusion proteins to allow for an additional mode of detection. The purification of these fusion proteins relies on protein A or protein G immobilized on sepharose, either in the form of suspended beads [193] or packed columns [20,

21]. In the case of zebrafish NITRs, NITR10- and NITR11-Fc fusion proteins were applied to the catfish B cell line 1G8 to study the details of receptor interactions with its unknown ligand on a target cell [193]. Fc fusion proteins made from TREM2A and TREM2B bind gram-negative as well as gram-positive bacteria and some yeast strains [20], whereas TREM3 fusion proteins did not. Bacteria identified via binding of TREM2-Fc fusion proteins to whole microorganisms are *Escherichia coli*, *Staphylococcus aureus* strains, *Candida guermundii* [20] as well as *Neisseria gonorrhoeae* [21]. The studies of TREM2A-Fc fusion proteins on *N. gonorrhoeae* were taken even further and TREM2A binding to membrane components of this pathogen was confirmed via Far Western dot blot [21]. The use of CHIR-Fc fusion proteins in an enzyme-linked immunosorbent assay (ELISA) technique to study CHIR binding to plate bound chicken IgY shows once more how versatile the use of these protein chimera is in regards to identifying immune receptor ligands and studying receptor ligand interactions [192].

Recombinant Fc fusion proteins are not the only means to generate soluble proteins as staining agents. In the study of NKR-P1 ligands, receptor tetramers were made containing only the NKR-P1 extracellular domains. The fusion proteins were produced and biotinylated in a bacterial expression system. The final tetramer formation relies on the use of streptavidin, in a 4:1 ratio of streptavidin to biotin, to link the biotinylated proteins together. The tetramers were then used as a staining agent to screen cells for ligand expression. These tetramers stained cells like NIH 3T3 and MNK-1 cells but not cells that were lacking osteoclast inhibitory lectin (Ocil/Clr-b) ligands on their surface [191].

Reporter cells are also a powerful tool commonly used in ligand identification, such as in the studies of NITRs [193], NKR-P1 [191], CHIRs [192] and TREMs [20, 21]. This approach relies on the generation of a chimeric receptor molecule containing the extracellular domains of the receptor molecule to be studied linked to the TM and cytoplasmic tail of the CD3ζ signalling adaptor. These chimeric receptor molecules are then expressed in inducible T cell

hybrids. These T cell hybrids have a nuclear factor of activated T cells (NFAT) transcription factor driven reporter gene stably integrated into their genome. The NFAT promoter is activated through the CD3ζ induced signalling cascade. Receptor clustering and consequent cellular activation can be induced via ligand binding or receptor cross-linking [191, 194].

In the studies of NITR binding to target cells, a 43-1 T cell hybrid was used. Activation of the stably expressed fusion receptor resulted in green fluorescent protein (GFP) expression in this reporter cell. Studies of eight different NITR-CD3ζ constructs expressed in these 43-1 cells revealed that only NITR11 constructs bound a ligand on catfish 1G8 B cells. None of the tested constructs bound to catfish 3B11 cells [193].

BWZ reporter cells, on the other hand, contain an NFAT driven β–galactosidase (lacZ) gene as reporter gene [194]. This particular reporter cell system has been used in ligand identification of CHIRs [192], NKR-P1 [191] and TREMs [20, 21]. BWZ reporter cells expressing CHIR-CD3ζ fusion proteins were incubated in wells coated with chicken antibodies to study IgY binding properties of CHIRs. Alternatively, co-incubation of receptor-CD3ζ chimeric protein expressing BWZ cells with target cells facilitated the identification of the NKR-P1 ligand. In detail, HEK 293T cells were transiently transfected with constructs encoding putative ligands. These transfected HEK 293T cells were co-incubated with BWZ cells and BWZ stimulation was evaluated via conversion of chromogenic or fluorogenic galacosidase substrates. Ocil/Clr-b was found to be the ligand for NKR-P1, whereas Clr-g and Clr-f were found not to be ligands for this NK cell receptor [191]. To examine TREM2 recognition of microorganisms, bacteria were co-incubated with the BWZ reporter cells expressing the TREM-CD3ζ fusion receptor. Chlorophenol red-β–D-galactopyranoside conversion in activated cells was used as a measure of receptor activation by these bacteria. TREM2A and TREM2B were confirmed to bind to *E. coli* and *S. aureus,* as well as to the outer membrane component dextran sulfate [20]. In additional studies, the BWZ reporter cell assay was used to confirm that TREM2A binds *N.*

gonorrhoeae and lipooligosaccharide purified from the *N. gonorrhoeae* outer membrane. BWZ TREM3 constructs did not activate the reporter cells when incubated with the whole bacteria or their outer membrane lipooligosaccharide [21]. The studies reviewed here illustrate how reporter cell assays can be used in conjunction with soluble staining agents to study immune receptor-ligand interactions, be it in screening cells for ligand expression, identifying ligand molecules or screening for soluble ligands. These techniques therefore present attractive options for the search of IpLITR ligands.

For the development of ligand identification methods, I selected several representative IpLITRs to generate recombinant proteins. These IpLITR representatives were cloned from those IpLITRs upregulated in cytotoxic lymphocytes upon alloantigen stimulation with 3B11 cells. They may therefore be involved in the allogeneic recognition of a target cell [165]. The IpLITR constructs chosen display a high degree of non-conserved amino acid changes within the putative MHC I binding region contained by the D1-D2 domains, which could confer variable binding properties between them. 3B11 cells have served as allogeneic stimulants for the upregulation of IpLITR expression. These cells therefore make excellent targets for the testing of IpLITR binding. In this chapter, I report the generation and expression of several IpLITR-Fc fusion constructs and describe the results from initial attempts to use these constructs as staining agents to screen cells for ligands. I also report the generation of IpLITR-CD3ζ fusion constructs for expression in BWZ reporter cells. The expression of a putative ligand on a cell that does not endogenously express these molecules could serve as an option to test for putative IpLITR ligands directly. For this reason, I also describe the results of experiments designed to express catfish MHC I complexes in HEK 293T cells. The surface expression of MHC I complexes depends on many steps, two of which are proper protein folding and the presence of a peptide loaded onto the MHC I α1 and α2 domains. Cold temperatures can aid in the surface expression of MHC I, even if the α chain is not loaded with an appropriate peptide [195]. For surface expression of channel catfish MHC I, I

therefore tested the incubation of transfected HEK 293T cells at standard incubation temperatures of 37℃ and at 27℃ after an initial incubation period at 37℃.

5.2 Results

5.2.1 Generation of soluble IpLITR-Fc fusion proteins

To generate soluble fusion proteins as staining agents to screen target cells for ligand expression, the D1 and D2 domains from IpLITR 2.0, 2.2, 2.3a, 2.6b and 1-3 were cloned into the pFUSE-mIgG3-Fc2 vector. This cloning strategy adds a mammalian IL2 secretion signal N-terminally and C-terminal human Cγ2 and Cγ3 domains of IgG3 (Fig. 5.1). The constructs were transfected and the proteins expressed in HEK 293T cells. The fusion proteins were predicted to be secreted into the culture supernatant. All constructs were confirmed via DNA sequencing.

Another set of constructs were made by cloning the IpLITR-Fc fusion constructs into the pDisplay vector (Fig. 5.2). This vector adds an HA-tag N-terminally to the fusion protein and an Ig κ leader peptide to facilitate secretion of the fusion proteins into the cell culture supernatant.

Following transfection into HEK 293T cells, the HA epitope tagged IpLITR-Fc fusion proteins generated from IpLITR 1-3, 2.0, 2.2, 2.3a and 2.6b were detected in whole cell lysates by Western blot with an anti-HA-HRP antibody (Fig. 5.3 top blot). In all experimental repeats, HA-IpLITR 1-3–Fc was the only construct detectable in the cell culture supernatant following 48 hours of incubation post-transfection (Fig. 5.3 bottom blot).

Despite the various antibodies used in an attempt to detect the Fc domains of the fusion proteins, untagged IpLITR-Fc fusion proteins could not be detected in transfected HEK 293T lysates or supernatants. Therefore all further studies were executed with the epitope tagged fusion proteins.

5.2.2 Examination of whether crude soluble IpLITR-Fc fusion proteins bind to catfish B cells

I tested the soluble IpLITR-Fc fusion proteins as a screening agent on catfish B cells predicted to express surface binding partners for these receptors (illustrated in Fig. 5.4). To this end, I applied crude lysates from transfected HEK 293T cells to 3B11 cells and incubated them for 30 minutes at 27℃. The 3B11 cells were then washed and stained for either the Fc region or the HA epitope tag of the chimeric protein. The constructs tested were derived from IpLITR 2.6b and IpLITR 1-3 and the HEK 293T lysates were applied to the B cells undiluted, in a 1/10 dilution, 1/50 dilution as well as a 1/100 dilution. Flow cytometric analyses of the 3B11 cells did not result in increased staining of the B cells above 12% when compared to 3B11 cells treated with lysate from non-transfected HEK 293T cells (Fig. 5.5). Fluorescence values for 3B11 staining experiments including lysates containing Fc fusion proteins generally stayed within 2% of the negative control at all tested concentrations.

5.2.3 Examination of whether IpLITR-Fc fusion proteins immobilized on fluorescent protein G sepharose beads bind to catfish B cells

Fluorescent protein G sepharose beads were incubated with lysates from HEK 293T cells transfected with HA-IpLITR 2.6b–Fc and HA-IpLITR 1-3–Fc at 4^{o}C and rotated for 24 hrs to allow for coating of the beads with the Fc fusion proteins. The beads were then spun down and the supernatant removed. The beads were washed three times and then stored at 4℃ in PBS with BSA. To test whether beads were coated with the fusion proteins, beads or supernatants were reduced in Laemmli buffer, separated by SDS-PAGE and then transferred to a nitrocellulose membrane. The membrane was then probed with an anti-HA-HRP antibody to confirm presence of the epitope tagged fusion protein (Fig. 5.6).

Under these conditions, IpLITR-Fc fusion constructs were detected on the beads and in the supernatant.

The coated beads were tested on two catfish B cell lines, 1G8 and 3B11 as outlined in Figure 5.7. The incubation of B cells with beads at different dilutions (1/1, 1/10, 1/50, 1/100) was performed at 27℃ and after gentle washes the cells were analysed by flow cytometry. The fluorescence of B cells incubated with Fc fusion protein coated protein G beads did generally not exceed 7% when compared to B cells incubated with control beads (Fig 5.8). There was also no concentration dependent trend when compared to beads that had been incubated with non-transfected HEK 293T lysates.

5.2.4 Generation of IpLITR-Fc-CD3ζ fusion proteins for expression in BWZ reporter cells

I generated four fusion receptors containing the D1 and D2 domains of IpLITR 2.6b or 1-3. One version of these constructs does not contain an epitope tag (Fig. 5.9). Another version does contain HA-epitope tags (Fig. 5.10).

5.2.5 Generation of epitope tagged catfish MHCI α chain and epitope tagged and untagged catfish β2m

In order to test catfish MHC I as a putative IpLITR ligand, I expressed the catfish MHC I α chain in conjunction with β2m in cells that do not endogenously express these molecules. I therefore cloned MHC I α chain and β2m from the catfish 3B11 cell line (as described in Materials and Methods) and then transfected these constructs into HEK 293T cells (Fig. 5.11 and Fig. 5.12). These proteins were produced in single and double transfection experiments as confirmed by Western blot analyses. The HA-tagged MHC I α chain size was detected at ~48 kDa, and the 3x FLAG tagged β2m detected at ~15 kDa (Fig. 5.13). For β2m, a second band above the protein band of expected size was observed, which may be a result

of glycosylation. Co-immunoprecipitation experiments were inconclusive and a strong association between the α chain and the β2m molecule could not be detected. Attempts to express these catfish molecules on the surface of HEK 293T cells have also not yielded conclusive results. The catfish MHC I α chain and β2m may just not be expressed at detectable and reproducible levels on HEK 293T cells, regardless of incubation temperature of 37℃ or 27℃.

In an attempt to switch the epitope tag, or remove it from the MHC I α chain and/or β2m molecules, respectively, I cloned the MHC I α chain into p3XFLAG-CMV™-9, and cloned β2m into pFUSE. The β2m intrinsic stop codon would avoid the addition of the human IgG3 Fc domains (Fig 5.12). The newly designed FLAG tagged MHC I α construct and the untagged β2m construct were expressed alone or in double transfections in HEK 293T cells. Western blot results from transfected HEK 293T whole cell lysates detected their expression (Fig 5.13). The 3xFLAG tagged MHC I α chain has an approximate size of 56 kDa and the untagged β2m was detected at ~13 kDa. Like the epitope tagged β2m, the Western blot of untagged β2m produced an additional band above the expected size. However, attempts to show an interaction between these FLAG-tagged catfish MHC I α chain and untagged β2m molecules and the examination of surface expression of these in HEK 293T cells were unsuccessful.

5.3 Discussion and conclusions

The use of purified Fc fusion proteins has aided in ligand identification studies in NITRs [193], CHIRs [192], and TREM-2 [20]. Therefore, soluble Fc fusion proteins were generated as an approach to screen target cells for the expression of IpLITR ligands. The production of untagged fusion proteins could not be confirmed due an inability to detect the Fc portion of the fusion proteins with the antibodies used in this study. This could be a matter of optimizing the detection procedure with the available anti-hIgG3 heavy chain antibodies.

HA-IpLITR-Fc fusion proteins were detected in HEK 293T lysates. However, not all constructs were secreted at detectable levels in the cellular supernatants. This could be an indirect effect of over-expressing these constructs in HEK 293T cells, which may lead to the accumulation of misfolded fusion proteins within the cells, thereby inhibiting their secretion and resulting in the inability to bind targets when isolated from the lysates in an unfolded form.

The observation that crude HEK 293T lysates containing HA-IpLITR-Fc proteins did not stain target cells is not too surprising. These lysates contain a complex mixture of cellular proteins that may block ligand-IpLITR interactions. More importantly, the concentration of IpLITR-Fc proteins within the lysates may just be too low in order to serve as an effective staining agent, which was difficult to estimate due to contaminating proteins. Purification and concentration of large amounts of these fusion proteins would be required to determine if higher concentrations of IpLITR-Fc fusion proteins do stain target cell lines such as 3B11. Media components such as BSA significantly interfered with previous fusion protein purification attempts, and as an alternative, these proteins could be produced in a media free of serum in HEK 293T cells in the future. The generation of an Fc fusion construct using a protein known to bind channel catfish B cells would serve as an important and necessary positive control. One molecule that has been shown to bind to catfish B cells through the generation of an Fc fusion construct is NITR11 [193]. If I would have had access to NITR11 Fc fusion proteins, I could have used it to better devise a B cell staining protocol. In the future, the procedure from Cannon *et al*. (2008) [193] can be applied as a useful strategy for the staining of catfish B cells with IpLITR Fc fusion proteins, if they do indeed interact with a protein on the surface of these target cells. Notably, there are major differences between the protocol used in this thesis and by Cannon *et al*. (2008). The Fc fusion proteins contain human IgG1 Fc domains and epitope tags at the junction between NITR and Fc domains, which excludes the possibility of N-terminal tag interference with ligand binding at distal residues of the recombinant protein. There is a greater variety of antibodies available

against human IgG1 in comparison to antibodies against mouse IgG3. The constructs are encoded in a different expression vector used in transfections of HEK 293T cells but in a different culture medium (RPMI) than that used in this thesis. The protein was purified from the culture supernatant with sepharose protein A beads. The proteins were quantified using an ELISA with human IgG1 as standard. A specified amount of protein (5µg / mL) was used in a staining procedure performed at 4℃ (not at 27℃, as in my protocol). Cells were washed in a similar manner as in my method but finally fixed in paraformaldehyde before analysis. All these different details in the procedure of generating and testing Fc fusion proteins are relevant and in the future this successful method could be used or adapted together with the NITR11 fusion constructs as a positive control to study IpLITR binding with soluble Fc fusion constructs on catfish B cells.

Fc fusion proteins can also be concentrated by coating Protein G beads with them. This strategy may also mimic more closely receptor clustering which often occurs during immune receptor activation in immune cells. This may also allow for better outcomes in studies of immune receptors that bind with low affinity but high avidity, a phenomenon common to the binding of immunoglobulins by Fc receptors [8]. Fusion protein tetramers also represent another strategy to apply several ligand binding domains in close proximity in ligand binding studies, such as in the examination of NKG2D [196] and NKR-P1 [197]. Techniques that bring many receptor binding domains in close proximity are therefore attractive in this aspect. The fact that I attempted to stain protein G beads only once and the lack of a positive control for the bead coating procedure, such as using the aforementioned NITR11-Fc fusion protein by the Litman group [193] or some purified IgG, limits my interpretation of whether or not the bead coating procedure is optimal or not. The presence of blocking proteins in the lysates may provide suboptimal conditions for the coating of sepharose protein G beads. The complete removal of all blocking proteins during the coating procedure of the sepharose protein G beads in the future would therefore also assure more efficient coating. One way to pre-purify the lysates would be to run

them through a size exclusion column, which is an available option since the size of the IpLITR-Fc fusion proteins is known (~50 kDa). This would already allow for the removal of a large amount of proteins and increased concentration of IpLITR-Fc fusion proteins. Again, one could try to coat a bead with NITR11-Fc fusion proteins and use these as a positive control in the optimization of the B cell staining procedure using coated beads in the future.

There are many different IpLITRs expressed by catfish immune cells. However, the constructs that I tested contained putative ligand binding domains of only two representatives; IpLITR 2.6b and IpLITR 1-3. Different IpLITRs may recognize different ligands or fulfill otherwise separate functions. It is therefore difficult to draw definitive conclusions for all IpLITR molecules from the inconclusive binding experiments of two representatives performed in these preliminary experiments. In the studies of CHIRs, only certain receptors from this multigene family demonstrated chicken IgY binding. The ligands of other members of the CHIR family are still unknown [60]. Moreover, the ligands of some members of the mammalian LILR family of receptors remain to be identified despite their discovery more than a decade ago and the many approaches available [31]. Whether the D1 and D2 domains of IpLITR 2.6b and/or IpLITR 1-3 bind any ligand on catfish B cells or any catfish target cell is unknown. Yet, these two constructs were cloned from those IpLITRs upregulated in cytotoxic lymphocytes upon alloantigen stimulation with 3B11 cells. Therefore, they may be involved in the allogeneic recognition of target cells [165]. such as the 3B11 cells, which were used in these studies. To develop ligand identification methods, I used two representative IpLITR constructs that display a high degree of polymorphism regarding their D1-D2 domains. The non-conserved amino acid residues in the putative MHC I binding site could result in differential binding of the same ligand. Moreover, several different ligands could be recognized by IpLITRs. The possibility that the IpLITR 2.6b and IpLITR 1-3 do not bind a ligand on catfish B cells can therefore not be excluded. Despite the lack

of target cell staining, I have taken important initial steps in developing and testing various methods for the identification of IpLITR ligands.

Many immune receptors also form dimers in order to function, be it homodimers such as NKG2D and FcγRIIA or heterodimers like CD94/NKG2A. Crystallization studies of one member of the catfish NITR family showed that these receptors form homodimers. They thereby create a ligand-binding surface crucial for receptor specificity [193]. FcγRIIA homodimers are formed via non-covalent interactions. Formation of these dimers appears to be critical for the signalling capabilities of the receptor [198]. A recent discovery indicates that putative stimulatory IpLITRs form homodimers as well as heterodimers [185]. Homodimerization was observed by Western blot analysis of whole cell lysates under non-reducing conditions and confirmed by immunoprecipitation of HEK 293T cells transfected with various epitope tagged IpLITR constructs. The stimulatory IpLITR types studied were IpLITR2.0, IpLITR2.3a and IpLITR2.6b. While IpLITR2.3a and IpLITR2.6b form homodimers, IpLITR2.0 does not. Heterodimer formation was observed for IpLITR2.0/IpLITR2.3a, IpLITR2.0/IpLITR2.6b and IpLITR2.3a/IpLITR2.6b, and these homo- and heterodimeric receptor complexes may contribute to their immunoregulatory properties in a manner like that suggested for FcγRIIA [198]. This notion is supported by the observation that IpLITR dimerization seems to be based on non-covalent interactions. The formation of homodimers as well as heterodimers may play a role in ligand binding as described for FcγRIIA homodimers, where the formation of a proper ligand binding interface seems to be dependent on non-covalent dimer formation between the extracellular D2 domains [185]. Therefore, the formation of homo- and heterodimers could also be crucial in order to observe binding to IpLITR receptors. While the formation of such dimers may not be stabilized within soluble fusion proteins, dimers may be formed on the surface of transfected cells. If this were the case, reporter cells expressing IpLITR constructs would be an attractive option for future ligand binding studies.

A reporter cell assay provides the advantage that clustering of the fusion receptor molecules following ligand binding. Clustering of immune receptors occurs in the cells in which they are naturally expressed as a requirement for efficient ligand binding and subsequent cellular signalling [199]. Moreover, the signal readout from a reporter cell such as the BWZ reporter cell is highly amplified and provides a sensitivity that exceeds other ligand screening methods. Figure 5.14 illustrates the function of the BWZ reporter cell, which relies on the activation of an enzyme cascade culminating in LacZ expression. Fusion receptor constructs containing the IpLITR 2.6 and 1-3 putative ligand binding domains have been produced and reporter cells expressing these constructs can now be generated using these constructs.

I cloned MHC I α chain and β2m from catfish B cells. The second band observed in β2m blots, which does not correspond to the expected size of the β2m molecule, may be a glycosylated form of β2m. The expression of the MHC I complex is quite intricate. It requires that the β2m and the α subunit assemble. An additional requirement is that the peptide binding grove of MHC I is loaded with an antigen. I have tested only one cloned MHC I α allele. However, the α1 and α2 subunits of MHC I are highly variable to allow for the display of a variety of antigens, making the genes encoding the MHC I α chain highly polymorphic. The tested MHC I α protein may be unable to load any peptide from HEK 293T cells. Interestingly, incubating the murine lymphoma mutant cell line RMA-S promotes MHC I assembly at reduced temperature (19–33°C). At these temperatures high levels of cell surface expression of H-2/β2m complexes not presenting endogenous antigens have been described. These complexes are labile at 37°C [195]. The resulting hypothesis that lower temperatures may stabilize the channel catfish MHC I complex and promote surface expression at catfish physiological temperature in HEK 293T did not hold true. Under no conditions tested did the MHC I complex display consistent or significant surface expression. Co-immunoprecipitation of MHC I α and β2m was unsuccessful, further supporting

that the association between MHC I α and β2m is either non-existent or very weak in HEK 293T cells. To express catfish MHC I on the surface of a cell that does not intrinsically express it, the expression system of choice could be an insect cell line. These cell lines do not require culturing equipment such as CO_2 incubators. They can be incubated at room temperature which may promote surface expression of catfish MHC I at a temperature closer to the catfish physiological temperature. This may also assist in proper protein folding.

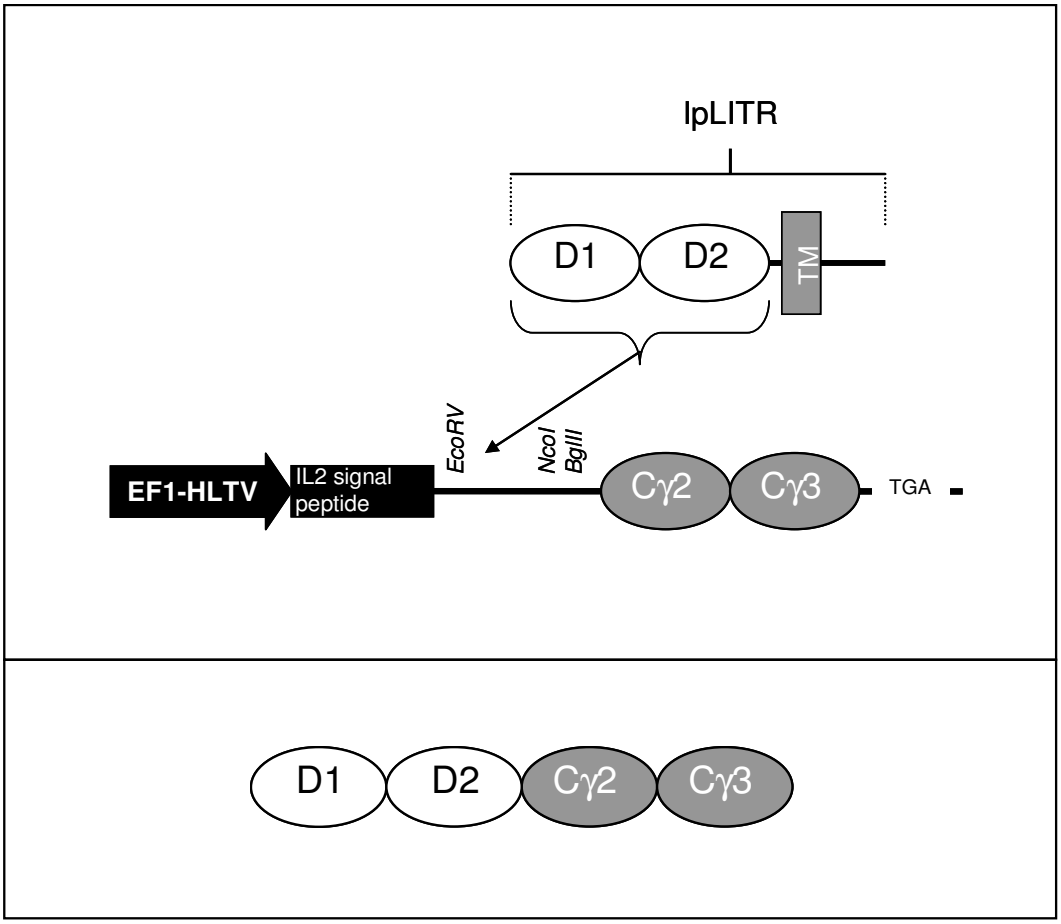


Figure 5.1. IpLITR-Fc fusion protein for expression and detection in HEK 293T cells. IpLITR D1 D2 domains were cloned into the pFUSE-mIgG3-Fc2 vector using EcoRV and NcoI or BglII restriction sites. The expression of the recombinant protein is driven from a composite EF1-human T-lymphotropic virus promoter. The resulting construct has an IL2 leader signal which allows for fusion protein secretion from mammalian cell lines.

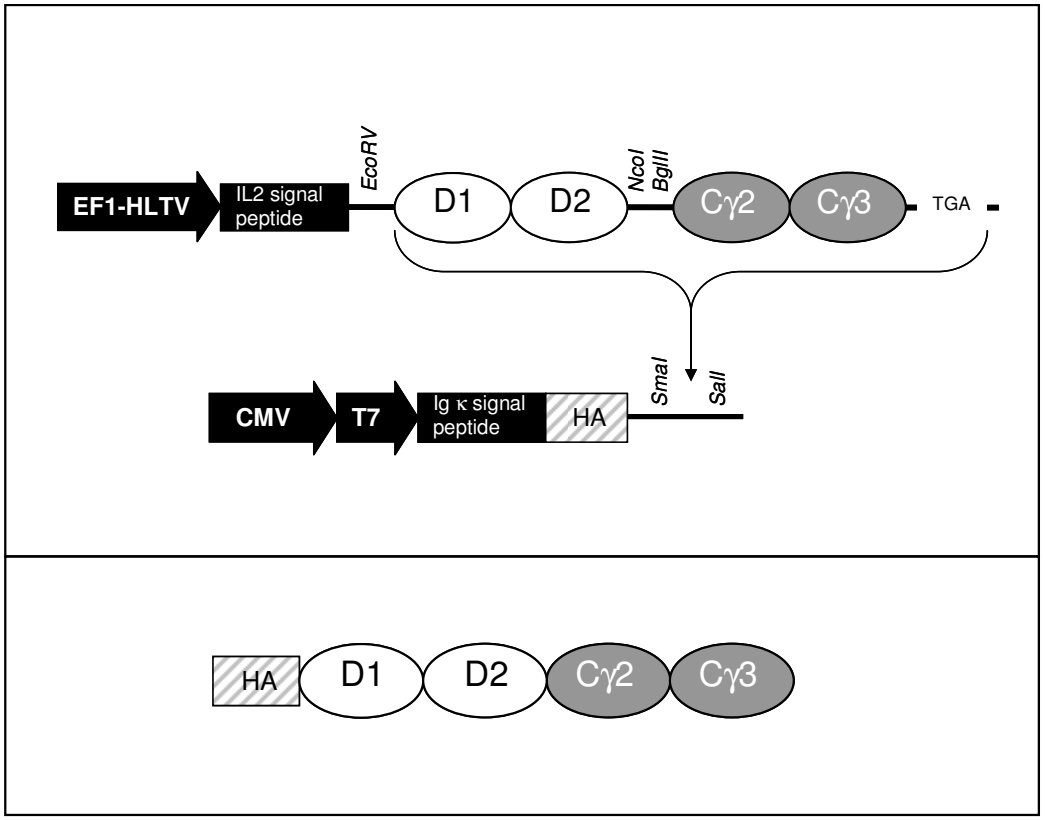


Figure 5.2. HA-IpLITR-Fc fusion protein for expression and detection in HEK 293T cells. IpLITR-Fc fusion constructs generated in the pFUSE system were cloned into the pDisplay™ vector SmaI and SalI restriction sites. The vector contains the human CMV immediate-early promoter and a mammalian TM following the Sal I site (TM not shown). The stop codon after the Cγ3 domain terminates translation past the Cγ3 C-terminus. The resulting construct has an HA epitope tag and an Ig κ leader signal. The signal peptide allows for secretion of this recombinant protein into the culture supernatant of a mammalian cell line.

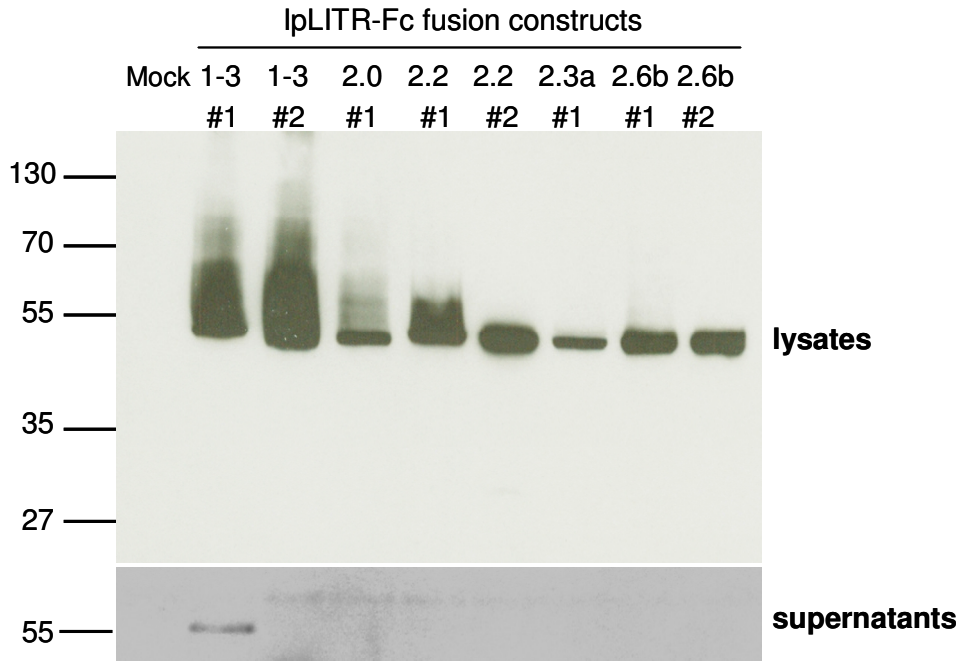


Figure 5.3. Western blot of HEK 293T whole cell lysates and culture supernatants transfected with IpLITR-Fc fusion constructs. Twelve and a half microlitres of reduced lysate samples and 2 µL of reduced cell culture supernatant were loaded into the appropriate wells of the SDS-PAG. The samples were separated on a 10% SDS gel under reducing conditions. The proteins were transferred onto a nitrocellulose membrane and detected with an anti-HA-HRP antibody.

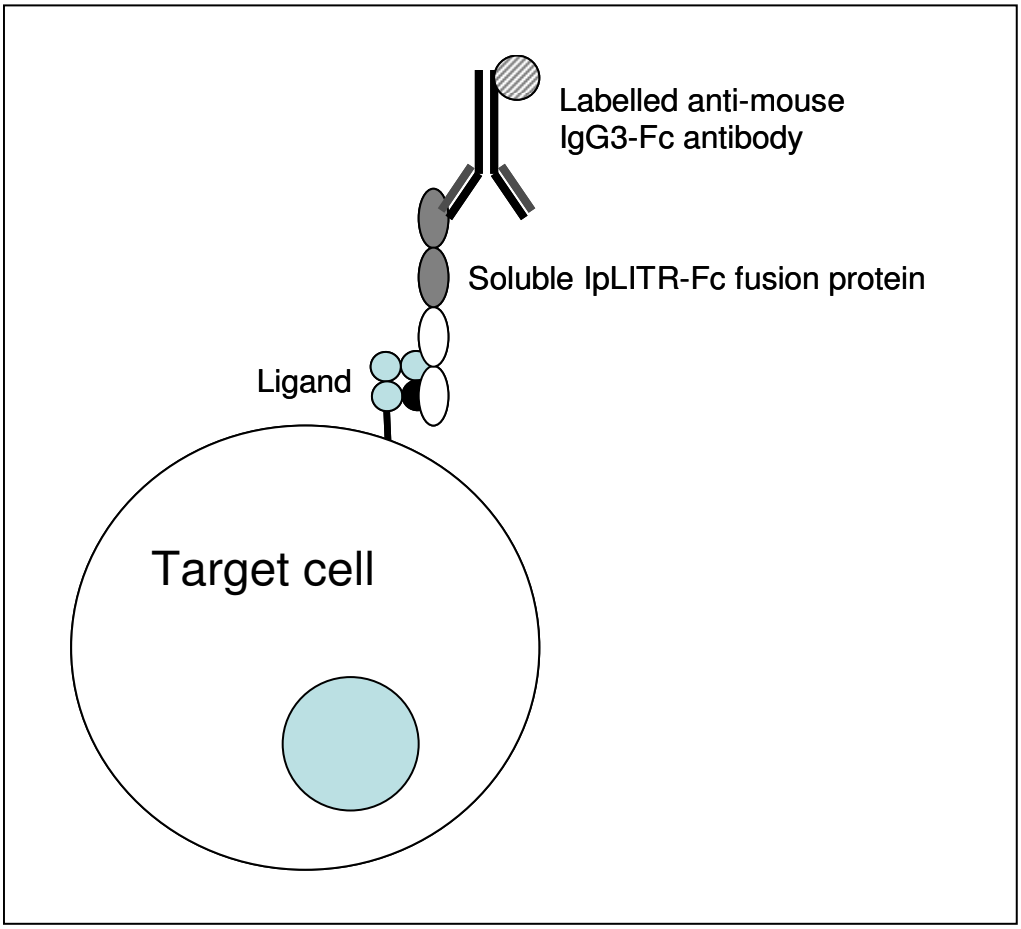


Figure 5.4. Soluble IpLITR-Fc fusion proteins as staining agents to screen target cells for ligands. A solution containing IpLITR-Fc fusion proteins is incubated with target cells that express IpLITR ligands. After gentle washing, a labelled anti-mouse IgG3 antibody that recognizes the Fc portion of the recombinant IpLITR protein is used to stain the cells with IpLITR constructs bound to their surface. To detect IpLITR binding the target cells are analyzed using flow cytometry.

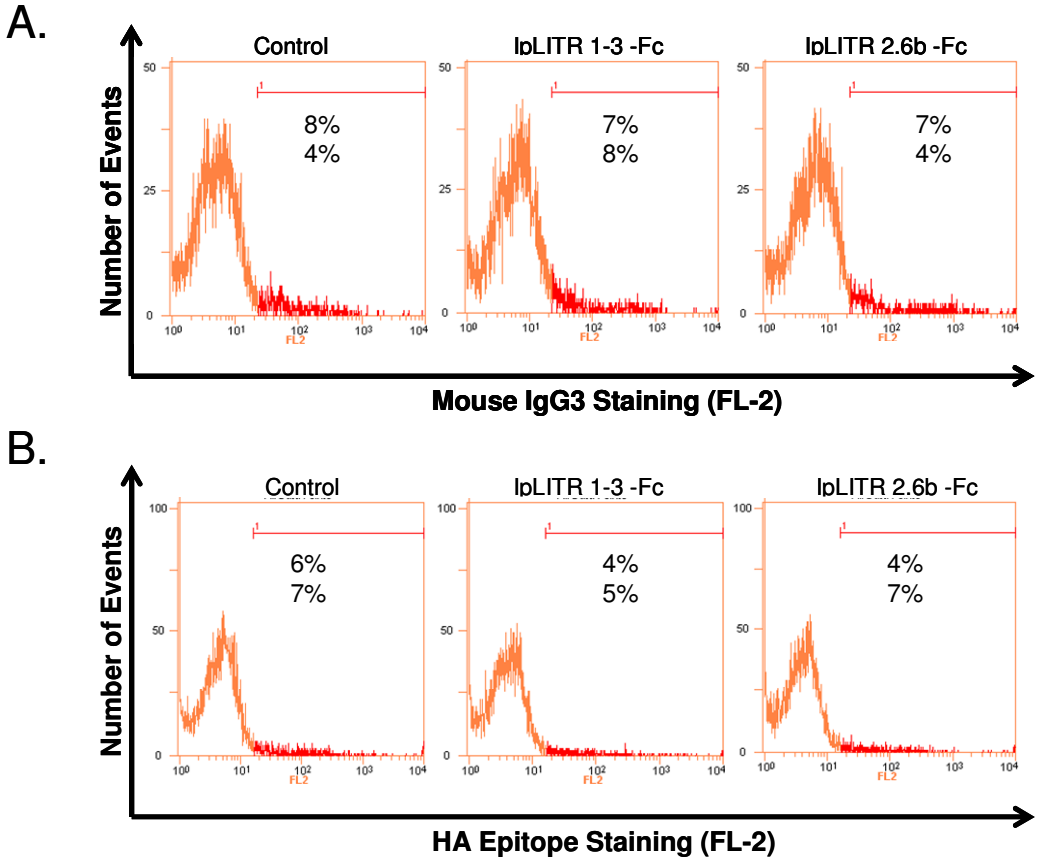


Figure 5.5. HA-IpLITR-Fc proteins from crude whole cell lysates of transfected HEK 293T cells do not bind to 3B11 cells. HEK 293T cells were transiently transfected with HA-IpLITR 1-3 or 2.6b, and 3B11cells were incubated with undiluted or 1:10 or 1:100 dilutions of the respective whole cell lysates. Non-transfected HEK 293T lysates served as control. Displayed are representative results for all tested dilutions: lysate dilution of 1:100 for (A) and 1:10 for (B). After gentle washing the cells were stained with a PE conjugated anti-mouse-IgG3 antibody. The cells were then analyzed on a flow cytometer (A). The same procedure was followed on 3B11 cells using a primary anti-HA antibody and a secondary anti-mouse-PE antibody (B). The results for two independent sets of experiments are shown as (%) in the panels and the top row in each panel correspond to the representative data displayed. In all staining experiments with crude lysates, staining never exceeded 12% in comparison to negative controls throughout all tested lysate concentrations.

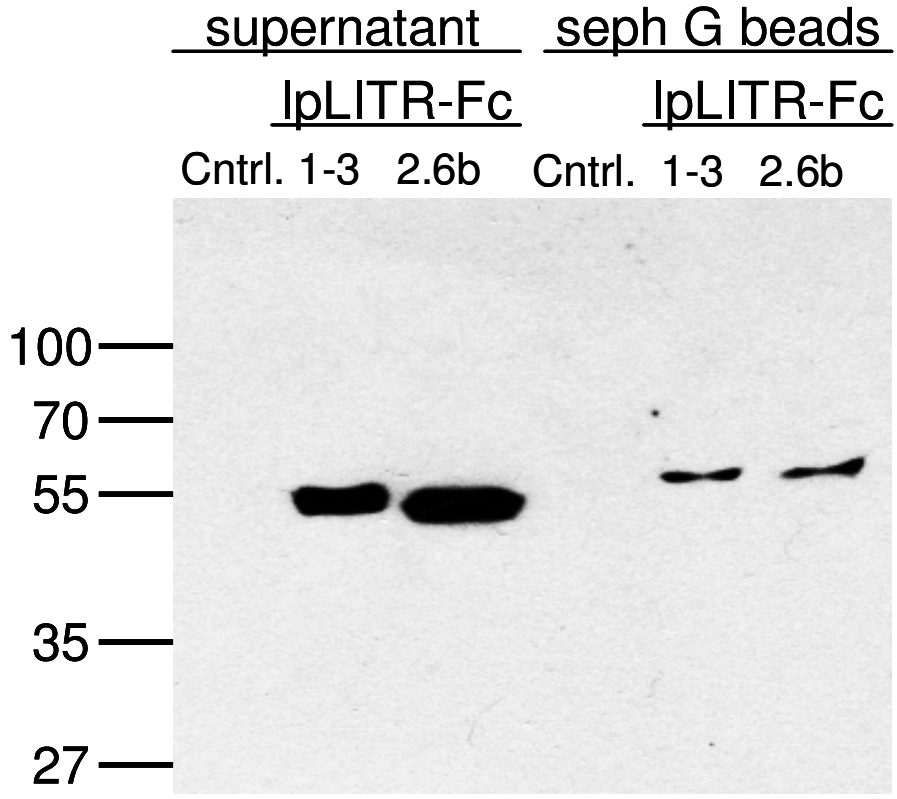


Figure 5.6. Western blot of sepharose protein G beads incubated with HEK 293T whole cell lysates transfected with IpLITR constructs. HEK 293T cells were transiently transfected with HA-IpLITR 1-3 or 2.6b and whole cell lysates were obtained. Lysates from non-transfected HEK 293T cells served as control. Sepharose protein G (seph G) beads were incubated with the lysates and gently washed. The proteins on the beads as well as in the bead supernatant (before washes) were separated on a 10% SDS-PAG under reducing conditions. The proteins were transferred onto a nitrocellulose membrane and detected with an anti-HA-HRP antibody.

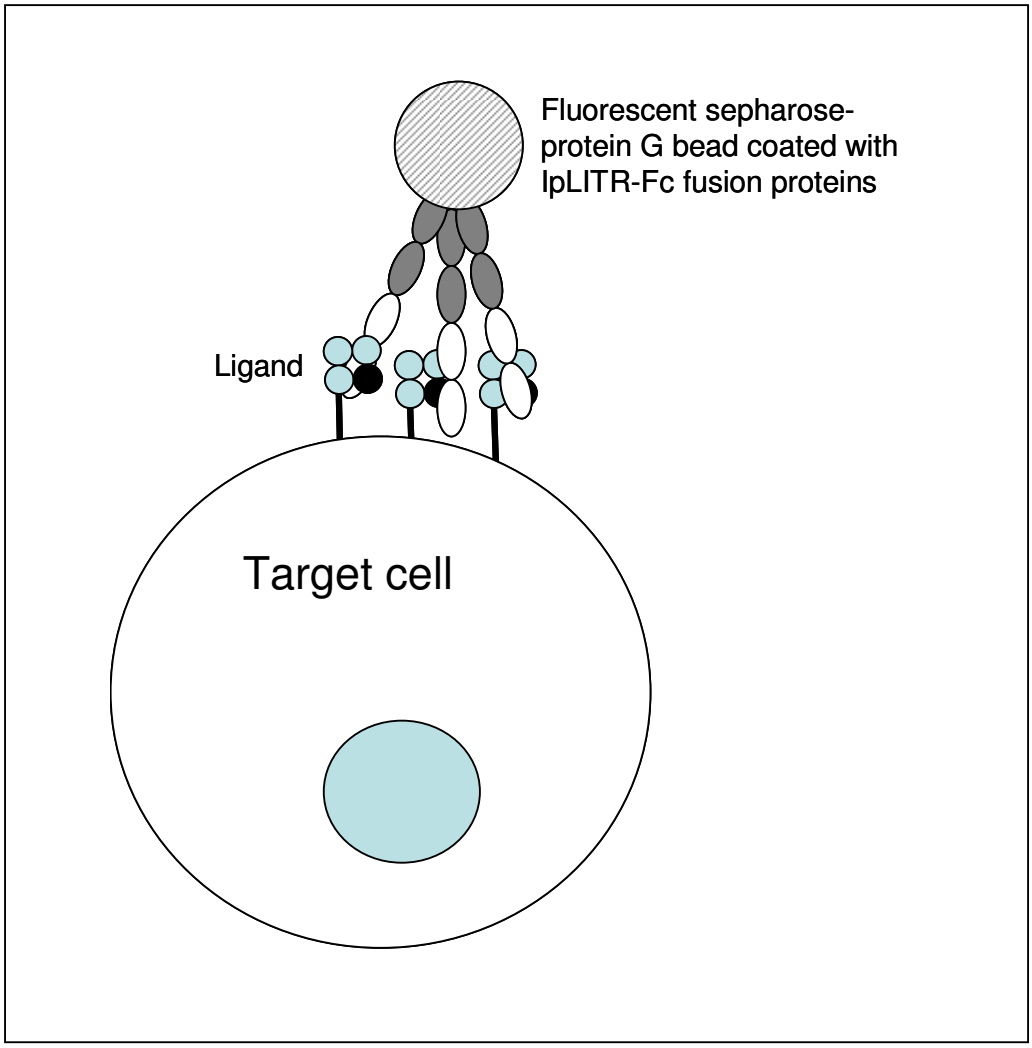


Figure 5.7. Schematic of IpLITR-Fc fusion proteins immobilized on beads as staining agents to screen target cells for ligands. Fluorescent protein G sepharose beads are coated with IpLITR-Fc fusion proteins and incubated with target cells that express IpLITR ligands. After gentle washing, IpLITR binding to the target cells is analyzed using flow cytometry.

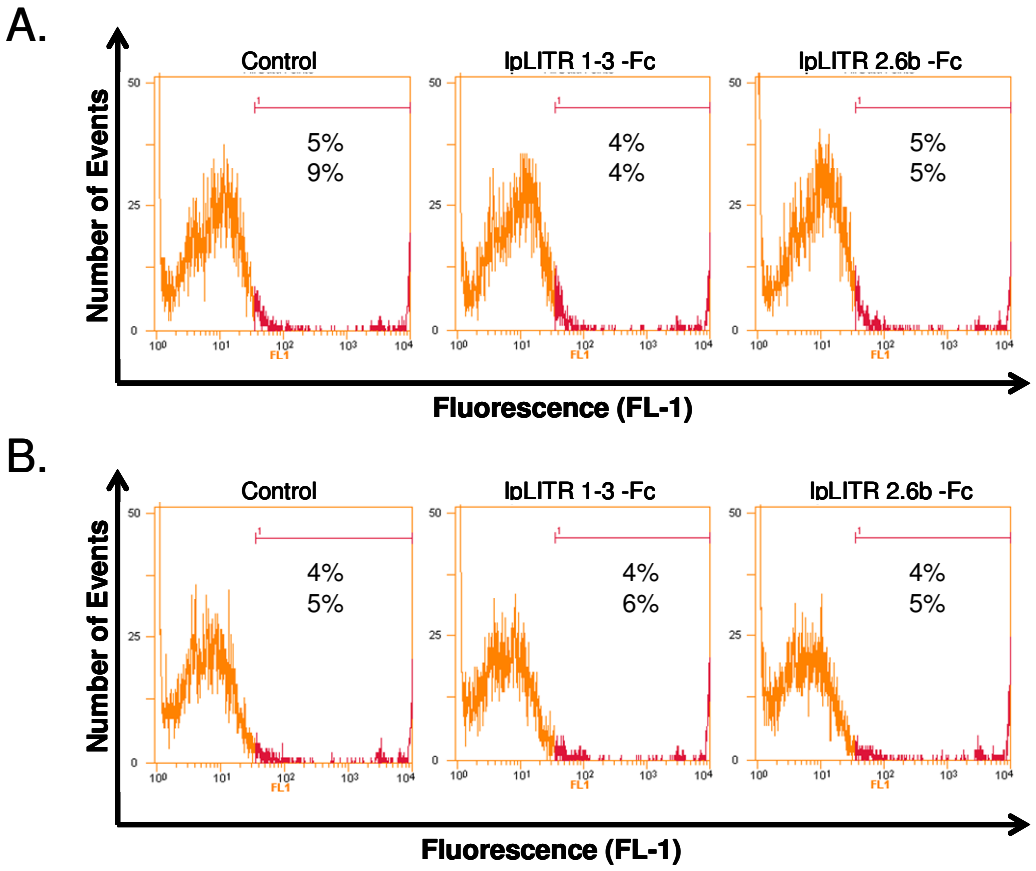


Figure 5.8. HA-IpLITR-Fc coated protein G beads do not bind to catfish B cell lines. HEK 293T cells were transiently transfected with HA-IpLITR 1-3 or 2.6b and whole cell lysates were obtained. Lysates from non-transfected HEK 293T cells served as control. Sepharose protein G beads were incubated either undiluted or at dilutions of 1:10, 1:50 or 1:100 with the 3B11 cells. After gentle washing 3B11 cells were incubated with the respective beads. After washing, cells were analyzed on a flow cytometer (A). The same procedure was used on 1G8 cells (B). Both results shown are from experiments performed at a 1:100 bead dilution as representative of all dilutions tested, which showed the similar results. The results of two staining experiments are shown as (%) in the panels and the (%) in the top row of each panel correspond to the representative data displayed. In staining experiments with coated protein G beads, staining stayed below 7% in comparison to negative controls throughout all tested lysate concentrations.

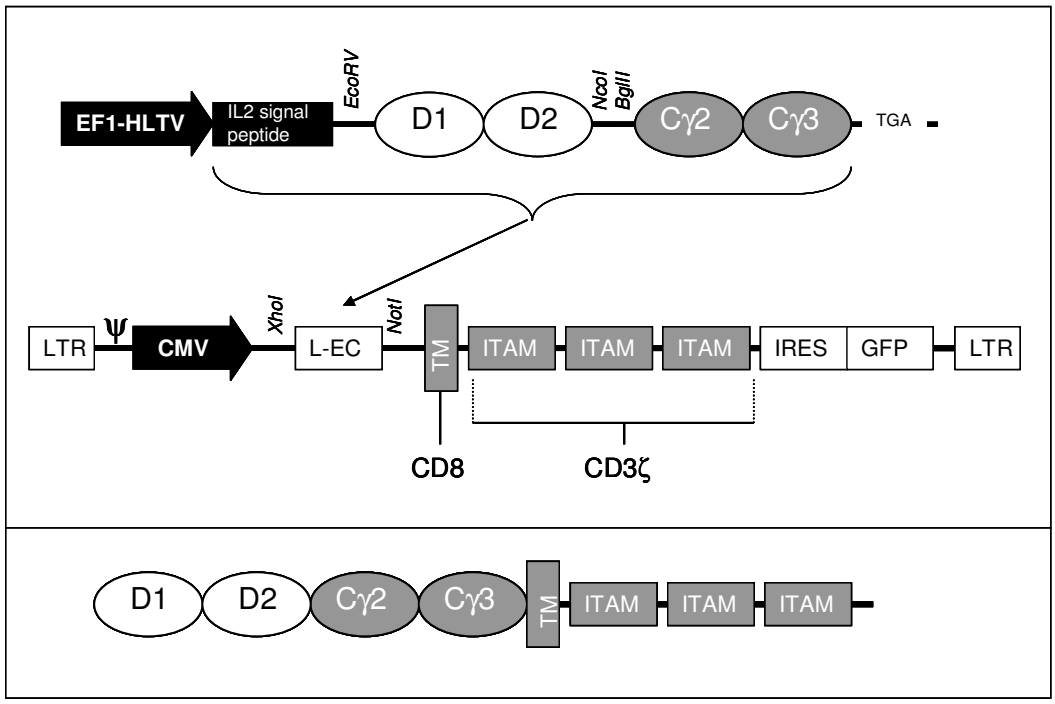


Figure 5.9. Chimeric receptor for surface expression in BWZ reporter cells. IpLITR-Fc constructs generated in the pFUSE system were cloned into the BWZ type I vector using NotI and XhoI restriction sites. The resulting construct has a mammalian IL2 leader signal, which allows for surface expression of this recombinant catfish protein in mouse BWZ cells, a single TM from mouse CD8 and the cytoplasmic tail of mouse CD3ζ. The BWZ type I vector also contains a GFP gene, which allows monitoring of transfection/infection efficiency, a retroviral packaging signal and LTR sites that, among other things, promote insertion of the depicted vector region into the host cell genome. Transfection of a retroviral packaging cell line with this BWZ type I vector will result in the production of retroviral particles containing the depicted part of the BWZ type I vector. These retroviral particles can then be used to infect BWZ cells, which can be selected and monoclonal cell lines can be made for use as reporter cells.

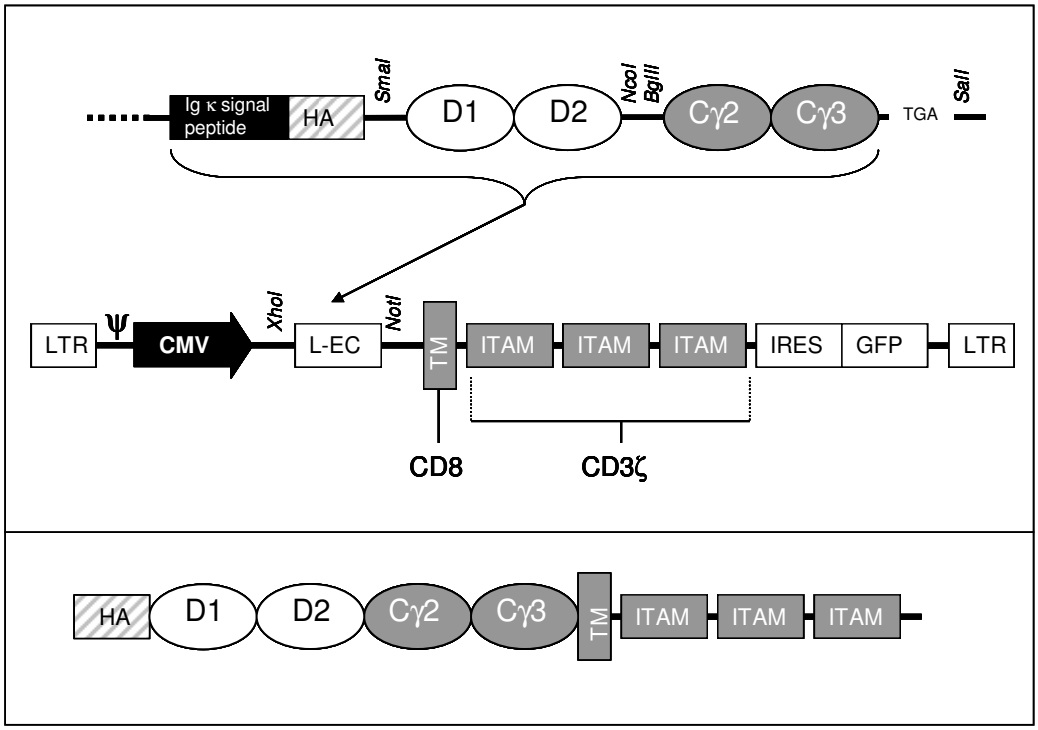


Figure 5.10. Epitope tagged chimeric receptor for surface expression in BWZ reporter cells. HA-IpLITR-Fc constructs generated in the pFUSE and pDisplay™ vector systems were cloned into the BWZ type I vector using NotI and XhoI restriction sites. The resulting construct has a mammalian IL2 leader signal, which allows for surface expression of this recombinant catfish protein in mouse BWZ cells, an HA epitope tag, a single TM from mouse CD8 and the cytoplasmic tail of mouse CD3ζ. The BWZ type I vector also contains a GFP gene, which allows monitoring of transfection / infection efficiency, a retroviral packaging signal and LTR sites that, among other things, promote insertion of the depicted vector region into the host cell genome. Transfection of a retroviral packaging cell line with this BWZ type I vector will result in the production of retroviral particles containing the depicted part of the BWZ type I vector. These retroviral particles can then be used to infect BWZ cells, which can be selected and monoclonal cell lines can be made for use as reporter cells.

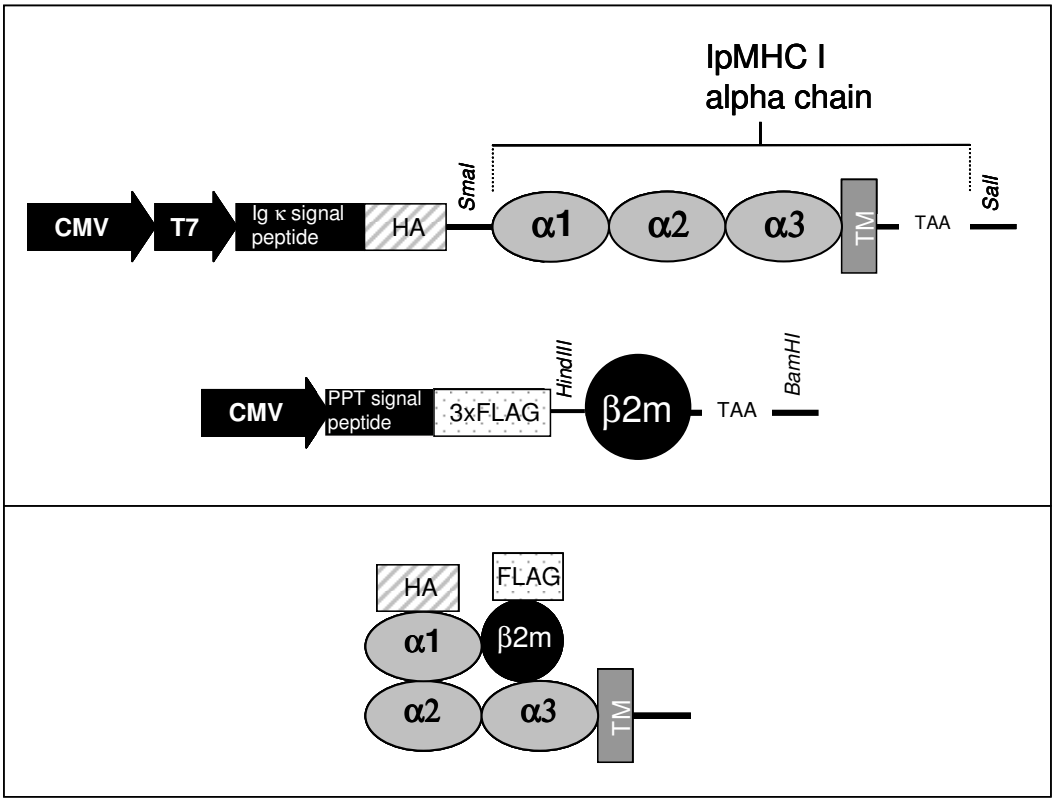


Figure 5.11. HA-tagged catfish MHC I α chain and FLAG-tagged β2m for surface expression and detection in HEK 293T cells. Catfish MHC I alpha chain was cloned into the pDisplay™ vector using SmaI and SalI restriction sites. The vector contains a cytomegalovirus promoter (CMV) and a mammalian TM following the Sal I site (TM not shown). The MHC I alpha chain stop codon terminates translation at the alpha chain C-terminus. The resulting construct has an N-terminal HA epitope tag and the mammalian Ig κ leader signal. Catfish β2m was cloned into p3XFLAG-CMV™-9 using HindIII and BamHI restriction sites. The vector also contains a CMV promoter driving the expression of the resulting construct with an N-terminal 3xFLAG tag and a mammalian preprotrypsin (PPT) leader signal.

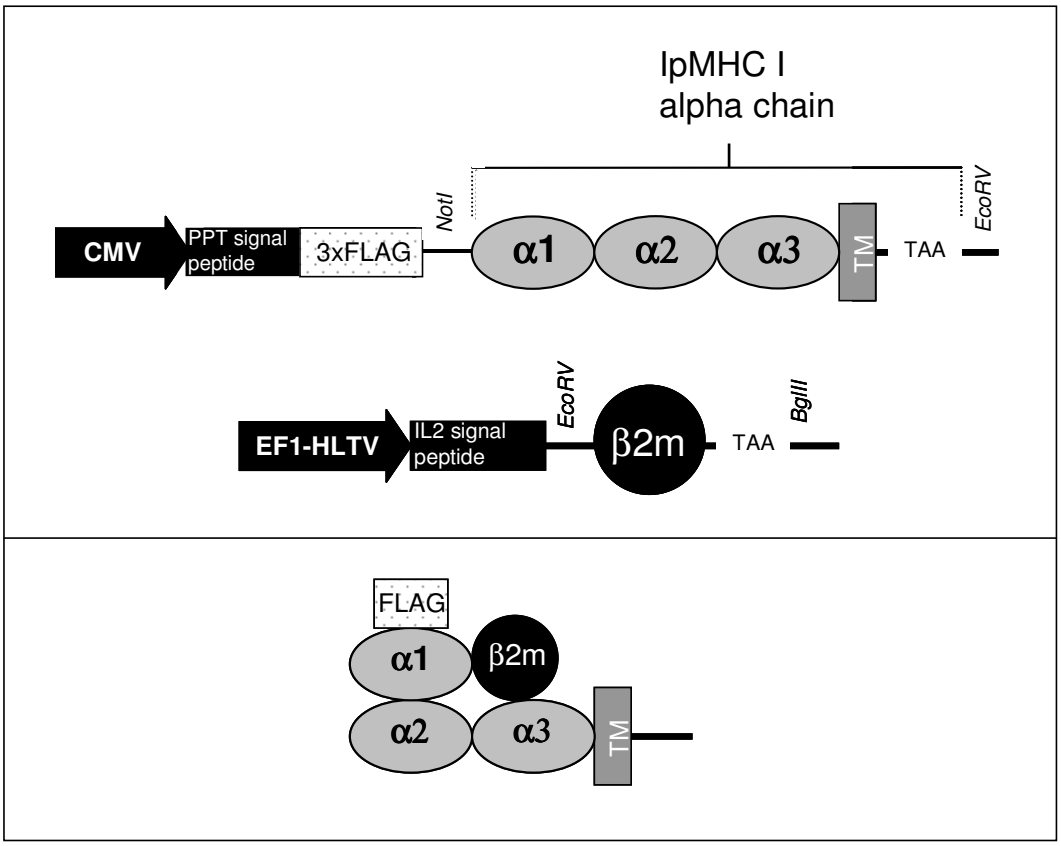


Figure 5.12. FLAG-tagged catfish MHC I α chain and untagged β2m for surface expression and detection in HEK 293T cells. Catfish MHC I α chain was cloned into the p3XFLAG-CMV™-9 vector using HindIII and BamHI restriction sites. The vector contains a CMV promoter to drive expression of the cloned gene. The resulting construct has an N-terminal 3xFLAG tag and the mammalian preprotrypsin (PPT) leader signal. Catfish β2m was cloned into pFUSE™ using EcoRV and BglII restriction sites. The β2m stop codon terminates translation past the β2m C-terminus. The resulting construct has a mammalian IL2 leader signal.

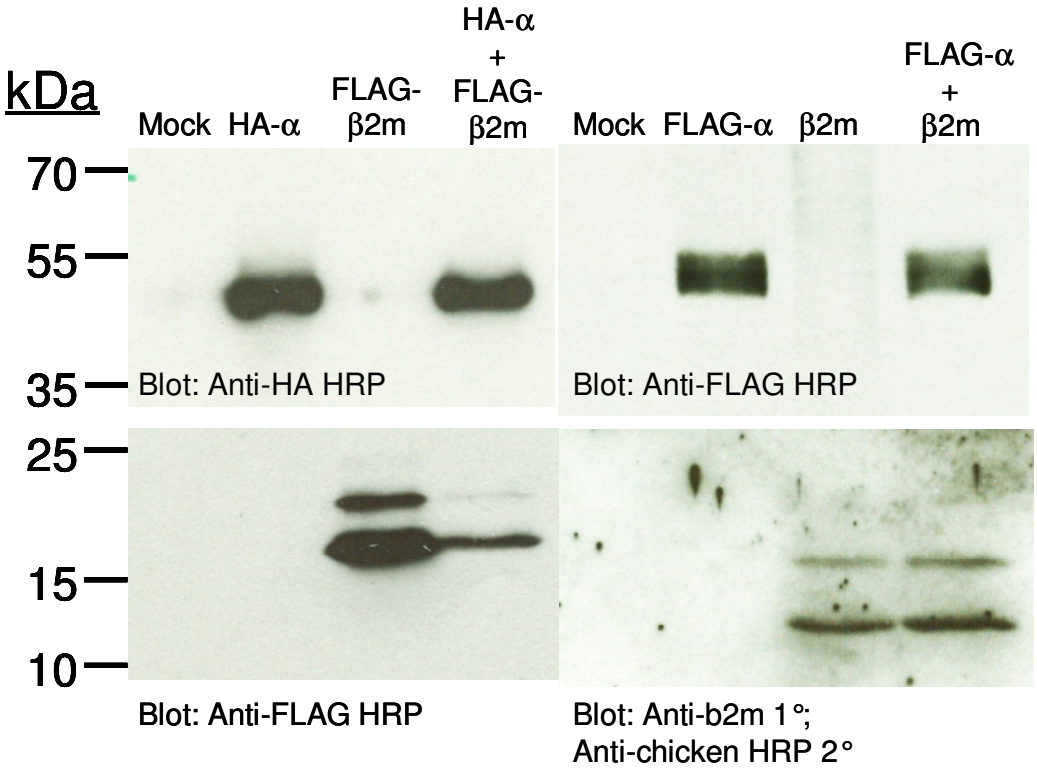


Figure 5.13. Western blot of HEK 293T whole cell lysates transfected with channel catfish MHC I α chain and/or β2m constructs. Twelve and a half microlitres of reduced whole cell lysates were loaded into the appropriate wells of an SDS gel. The lysates were separated on a 10% SDS gel under reducing conditions. The proteins were transferred onto a nitrocellulose membrane and detected with anti-HA-HRP antibodies, anti-FLAG-HRP antibodies or anti-catfish-β2m antibodies followed up with an anti-chicken-HRP antibody. Results are representative of three independent experiments.

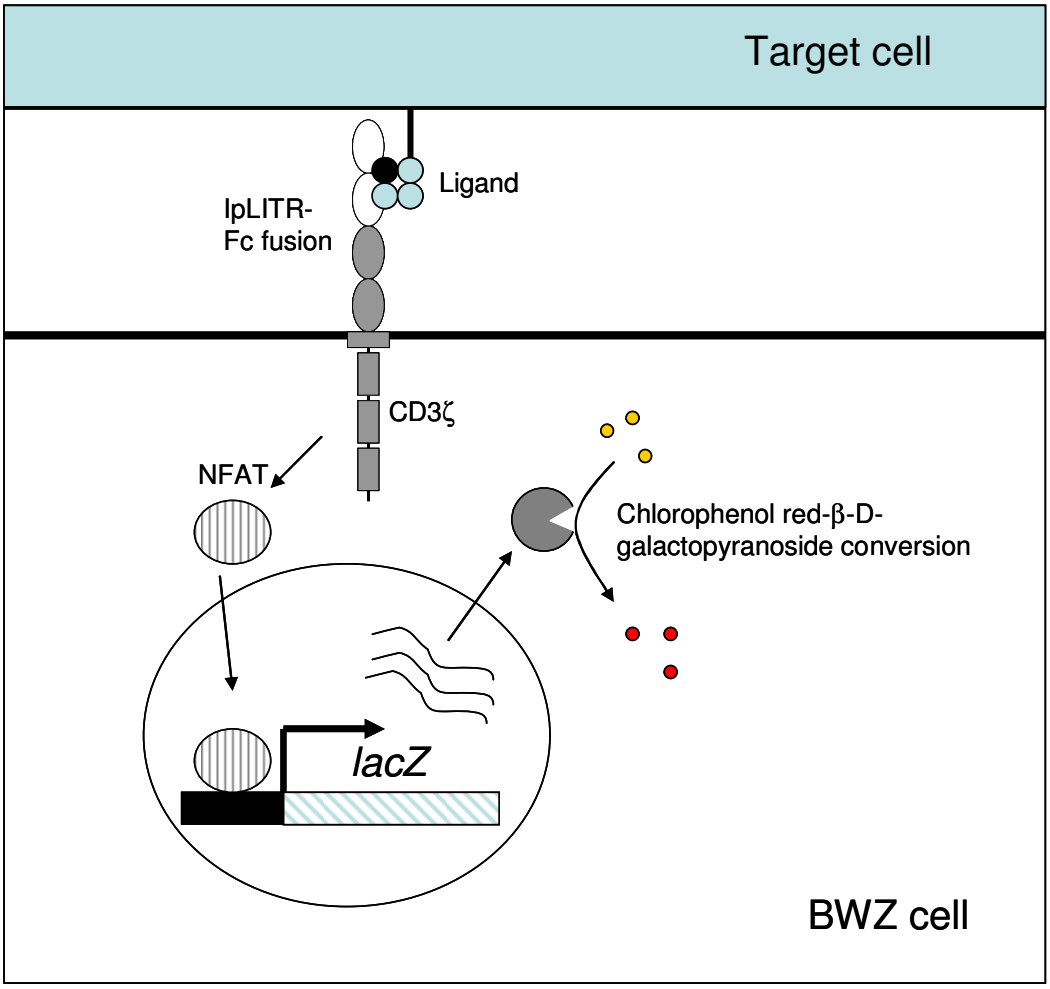


Figure 5.14. BWZ reporter cells function through the activation of a chimeric CD3ζ receptor molecule and expression of LacZ. BWZ stimulation can occur following incubation with target cells that express a ligand. When the ligand is recognized by the IpLITR D1D2 domains signalling events are initiated through the CD3ζ. intracellular domain. As a result, NFAT nuclear translocation and transcription of *lacZ* ensue. β-galactosidase activity can be examined through addition of an appropriate substrate, such as chlorophenol red-β-D galactoside whose conversion can be spectro-photometrically metrically monitored by formation of a red product.

CHAPTER 6
GENERAL DISCUSSION

6.1 Summary of findings

The discovery of IpLITRs marked an important step in further understanding the common origin and evolutionary history of innate immunoregulatory receptors [28]. While forty IpLITRs have been cloned, sequenced and subjected to phylogenetic analyses as well as homology modelling [28, 165] nothing was known of the functional significance of these novel teleost proteins and no tools were available for the studies of these molecules when I started my work on these molecules. The primary objectives of this thesis were to functionally characterize putative stimulatory IpLITRs regarding their signalling potential and to develop methods for the identification of IpLITR ligands. Since putative stimulatory IpLITRs are lacking signalling motifs I tested whether IpLITRs associate with teleost signalling adaptors that contain ITAMs. I showed that a representative stimulatory IpLITR indeed associates with ITAM containing signalling adaptors.

Association of fish immune receptors with signalling adaptors was first demonstrated for NITRs and DAP12 [183]. My results show that IpLITRs associate with other ITAM-containing signalling adaptors. Signalling via ITAM containing adaptors was first studied in mouse and human immune cells and also plays a role in immune receptor signalling in birds [200]. Moreover, ITAM-containing signalling adaptors FcRγ and CD3ζ have been identified in *Xenopus* [182], which indicates that they may also play a role in amphibian immune cell signalling.

To shed light on the IpLITR signalling potential, with no monoclonal antibodies against these catfish proteins at hand, I generated several epitope tagged putative stimulatory IpLITR constructs for expression in HEK 293T cells. I also generated epitope tagged teleost signalling adaptors and showed through co-immunoprecipitation experiments as well as flow cytometric analyses in co-

transfection experiments in HEK 293T cells, that stimulatory IpLITRs can associate with signalling adaptors and are expressed on the cell surface in association with FcRγ chain homologues. This discovery is in line with what is known for functional stimulatory immune receptors in other vertebrates [40-43, 115, 182-184, 192, 201] and indicates that putative stimulatory IpLITRs have the potential to be functional receptor molecules. Precisely, that FcRγ as well as CD3ζ can be recruited to the receptor, but only association with FcRγ homologues resulted in increased surface expression of IpLITR 2.6b, is an intriguing observation. It suggests that FcRγ homologues may be the functionally significant signalling adaptors under the tested conditions [186]. IpLITRs are expressed by various catfish leukocytes. They may therefore associate with CD3ζ-FcRγ dimers, as described for FcRs [189]. The expression of adaptor and IpLITR constructs in HEK 293T cells for association studies certainly is a limitation, since HEK 293T cells are not functional immune cells. Also, HEK 293T cells are not suitable to study further signalling events initiated by these teleost ITAM containing signalling adaptors.

In order to fully appreciate the role of IpLITRs in immune cell function, the knowledge of their ligands is of fundamental importance. I therefore generated a variety of recombinant proteins. These proteins contain putative IpLITR ligand binding domains and have the potential to serve in different experimental approaches to identify IpLITR binding partners. I took the first steps in developing methods to screen target cells for ligand expression. I generated IpLITR-Fc fusion proteins, and initial screens on cells, which evoke allogeneic killing by catfish cytotoxic cells [28, 165], did not show staining with the tested constructs under the specific conditions applied. Nonetheless, the first steps for future screening were made. Optimization of fusion protein purification, concentration and staining procedures can commence from here. In addition, IpLITR-CD3ζ fusion constructs were produced. These constructs can serve as fusion receptors in ligand screens when expressed in a BWZ reporter cell. I also wanted to express channel catfish MHC I on cells that do not naturally express

channel catfish MHC I. This process would generate target cells which allow testing for channel catfish MHC I complexes as ligands directly.

The generation of Fc-fusion constructs is a relatively simple strategy that allows for the screening of target cells for ligand expression. It has been successfully used in the studies of many immune receptors from various organisms such as mice [20, 21], birds [192] and fish [193]. The IpLITR fusion constructs for ligand screens have been generated. However, the purification of these proteins still requires optimization. Comparison of my methods with those used in the literature [20, 21, 193] indicate that purification and concentration of Fc fusion proteins may be critical in order to use the proteins as proper staining agents. The expression of these constructs under serum free conditions may help to detect and purify the proteins from cell culture supernatants. Fast protein liquid chromatography may also aid in purifying these proteins from HEK 293T lysates and/or supernatants. Coating of protein G sepharose beads, for use as a staining reagent, also has to be optimized. The B cell staining procedures were adapted from methods that have lead to successful ligand binding of Fc-fusion constructs in studies of different immune receptors [193]. However, these procedures also need to be further optimized regarding incubation periods and other conditions.

Reporter cell assays are often used in conjunction with soluble proteins to identify ligands or receptor binding to target cells [20, 21, 191-193]. These experiments have the advantage of giving dose dependent read-outs. They also allow for fusion receptor clustering on the reporter cell. This mechanism may be required for strong ligand binding and signal transduction [8]. I generated IpLITR-CD3ζ constructs for expression in BWZ reporter cells. However, the transfection of these T cell hybrids requires retroviral infection or nucleofection, two procedures that require time and optimization. Transfected cells will then have to be sorted or selected, and clonal stable lines can be developed from there.

To generate a target cell to test for a putative ligand specifically, a putative ligand can be expressed on a cell that does not naturally express it. This is a particularly attractive method, when the ligand is a protein complex that cannot be

tested in a soluble form. Prior sequence analyses and homology modelling indicate that there is a putative MHC I binding site in some IpLITRs [165]. For this reason, I cloned MHC I α chain and β2m molecules from channel catfish B cells. However, all attempts to express them as a complex on the surface of HEK 293T cells failed. This is not too surprising, since protein folding and proper association require appropriate temperatures. The expression of MHC I complexes is an intricate process that also requires loading with a suitable peptide onto the peptide binding groove of the MHC I α chain. HEK 293T cells may not generate peptides that can be loaded onto the cloned MHC I α chain of channel catfish tested in my experiments. The incubation temperatures of 37℃, required for optimal protein production in HEK 293T cells may also lead to misfolding of fish proteins. Insect cells may provide an alternative for the expression of proteins on their surface. These cell lines can be incubated at lower temperatures, similar to those for catfish cell incubation. This may aid proper folding and consequently surface expression. Also, protein association studies with tagged proteins can be a challenge. Tags sometimes interfere sterically with the association of these proteins. Therefore, a second cloning strategy was undertaken with untagged β2m, which also yielded inconclusive results in HEK 293T cells. Therefore, whether a target cell that expresses channel catfish MHC I can be made will have to be determined in future experiments.

6.2 Future directions

To further functionally characterize IpLITRs signalling, the receptors will have to be expressed in immune cells. Recent follow up work from the signalling adaptor association studies has therefore focussed on the development of a mammalian immune cell line such as RBL-2H3 (a rat basophilic cell line) stably expressing epitope tagged IpLITRs. Even though RBL-2H3 cells are not fish cells, many immunological signalling mechanisms are conserved among vertebrates. Therefore, they still are a promising system to gain insights on signalling of

catfish immune receptors and how this influences cellular effector functions. In particular, cytokine release, degranulation and phagocytosis are typical processes in activated immune cells, which can be studied precisely in this cell line [202]. For example, stimulatory IpLITRs can signal through signalling adaptors intrinsic to RBL-2H3 cells as well as catfish signalling adaptors. This was shown using cross-linking and phagocytic studies using antibodies targeting the HA epitope tags on the IpLITR constructs expressed on RBL-2H3 (unpublished data). This sets the stage to shed light on the precise signalling events following signal adaptor association, such as kinase recruitment to the adaptor and activation of pathways further downstream that then result in the observed degranulation and phagoctytic events.

With such a well established read-out from the activation of RBL cells following IpLITR activation, these cells can presumably also act as a reporter cell for use in ligand identification. An interesting approach in this follow-up work was the generation of chimeric catfish receptors that have the extracellular domains of IpLITRs, a mutated (neutral) TM and the intracellular domains of channel catfish FcRγ chain. The principal use of these chimeras could be as described for BWZ reporter cells. IpLITR-FcRγ expressing RBL-2H3 cells can be incubated with target cells and then analyzed for degranulation and cytokine release. Alternatively, a plate could be coated with putative soluble ligands and then incubated with IpLITR or channel catfish FcRγ fusion protein expressing RBLs. In addition, BWZ reporter cells can also be generated via nucleofection or retroviral infection using the constructs I generated (Chapter 5.1).

Other approaches can be taken to generate target cells expressing catfish MHC I, such as protein expression in insect cell lines. However, other ligands for IpLITRs should also be considered. For example, whether IpLITRs bind Ig can be tested by ELISA, reporter cells or staining of transfected cells expressing IpLITRs with Igs followed by flow cytometric analysis.

While relatively little is known about how fish immune receptors regulate immune effector functions, my work on IpLITRs has set the stage for future

studies. These studies will aim to further elucidate the ability of IpLITRs to regulate catfish immune cell functions such as phagocytosis, degranulation and cytotoxicity. These are processes that are crucially involved in the elimination of pathogens. The regulation of such immune effector functions through IpLITRs could therefore affect and control disease in the channel catfish. This is of particular concern for the catfish farming industry as there is a general desire to breed resistant livestock to assist in disease control within the field of aquaculture. Understanding the contribution of IpLITRs to channel catfish immune cell functions will also reinforce the relevance of the channel catfish as an immunological model. This model bridges our understanding of the evolution of the immune system between vertebrates, and can also find applications in the field of aquatic toxicology where the availability of channel catfish immune cell lines is a particular asset. Current collaborations, that utilize channel catfish B cell lines to study the effect of nanoparticles on fish immune cells, are a great example of how insights into channel catfish immune cell functions can help connect the applied field of environmental toxicology with immunology.

CHAPTER 7

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