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Biochemical and Functional Characterization of Inhibitory Leukocyte Immune-Type Receptors in the Channel Catfish (*Ictalurus punctatus*)

by

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ABSTRACT

A balance of intracellular signaling events control immune cell functions. In innate immunity, many of these signals are transmitted by subsets of cell surface proteins known as immunoregulatory receptors. Through their recognition of a diverse array of molecules these receptors effectively translate extracellular cues into immune cell responses. These effector responses are vital for the elimination of pathogens and the destruction of damaged and/or infected host cells. In general, the ability of immunoregulatory receptors to promote or abrogate cellular responses is directly linked with their ability to transmit stimulatory and/or inhibitory signals. These signals are responsible for the direct immune cell-mediated elimination of pathogens, such as viruses and bacteria, and simultaneously protect healthy host tissue from autoimmune reactions. Co-expression of stimulatory and inhibitory immunoregulatory receptor-types by immune cells is common in all vertebrates but the majority of the research into understanding these complex networks has focused on mammals. Recently, it has been shown that immune cells in non-mammalian vertebrates, such as teleost (i.e. bony fish), express larger repertoires of immunoregulatory proteins but evidence as to how they are involved in mediating cellular signaling and controlling effector responses remain unknown.

Channel catfish (*Ictalurus punctatus)* leukocyte immune-type receptors (IpLITRs) represent a large polymorphic gene family with members that possess the characteristics of putative stimulatory and inhibitory immunoregulatory proteins. The goal of my thesis work was to determine whether putative inhibitory IpLITR-types were bona fide functional receptors and to examine the signaling molecules/pathways involved in their activity. Using standard cellular transfection procedures, co-immunoprecipitations, site-

directed mutagenesis, and adaptation of a vaccinia virus-based expression system for displaying IpLITRs on the surface of freshly isolated mouse natural killer (NK) cells, I discovered that two putative IpLITR-types, termed IpLITR1.2a and IpLITR1.1b, are both functional inhibitory receptors. I also determined that these IpLITRs effectively diminish cellular responses using two different mechanisms. Specifically, both IpLITR1.1b and IpLITR1.2a abrogated NK cell-mediated killing using 'classical' inhibitory tyrosine-based signaling motifs within their cytoplasmic regions, which recruited inhibitory phosphatases. Unlike IpLITR1.2a, IpLITR1.1b has additional tyrosine residues encoded within the membrane proximal region of its cytoplasmic tail that are not contained within known inhibitory motifs. Interestingly, one of these unique tyrosines abrogated the killing response of NK cells. However, unlike what I showed for IpLITR1.2a this was via a phosphatase-independent mechanism. These results highlight the conserved aspects of immunoregulatory receptor signaling in vertebrates while simultaneously revealing subtle nuances that may contribute towards a greater understanding of cellular effector responses.

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List of Abbreviations

- ADCC antibody-dependent cellular cytotoxicity
- APC antigen presenting cell
- BCR B-cell receptor
- CD cluster of differentiation
- cDNA complementary DNA
- CAM cell adhesion molecule
- CEACAM carcinoembryonic antigen-related CAM
- chr chromosome
- CHIR chicken Ig-like receptors
- CLM CMRF35-like molecule
- Csk C-terminal Src Kinase
- Csw corkscrew
- CYT cytoplasmic
- D domain
- DICP diverse Ig domain-containing protein
- DMEM Dulbecco's modified Eagle's medium
- DN dominant negative
- DNA deoxyribonucleic acid
- D-PBS Dulbecco's PBS
- EAT EWS-activated transcript
- EC extracellular
- EDTA ethylenediaminetetraacetic acid
- ELISA enzyme-linked immunosorbent assay
- EST expressed sequence tag
- EWS Ewing's sarcoma
- FcR Fc receptor
- FCRL FcR-like
- GFP green fluorescent protein
- HA hemaglutinin
- HLA human leukocye antigen
- IgSF immunoglobulin superfamily
- IP immunoprecipitation
- IL interleukin
- ITAM immunoreceptor tyrosine-based activation motifs
- ITIM immunoreceptor tyrosine-based inhibition motifs
- ITSM immunoreceptor tyrosine-based switch motifs
- KIR killer cell Ig-like receptor
- KLRG killer cell lectin-like receptor
- LAIR leukocyte associated Ig-like receptor

- LILR leukocyte Ig-like receptor
- LITR leukocyte immune-type receptor
- LMIR leukocyte mono-Ig-like receptor
- LRC leukocyte receptor complex
- mAb monoclonal antibody
- MAPK mitogen-activated protein kinase
- MDIR modular domain immune-type receptors
- MHC major histocompatability complex
- MOI multiplicity of infection
- NCR natural cytotoxicity receptor
- NFAT nuclear factor
- NILT novel Ig-like transcript
- NITR novel immune-type receptor
- NK natural killer
- ocil osteoclast inhibitory ligand
- pAb polyclonal antibody
- PAGE polyacrylamide gel electrophoresis
- PBS phosphate buffered saline
- PCR polymerase chain reaction
- PEI polyethylinime
- PI3K phosphatidylinositol 3-kinase
- PIP_3 phosphatidylinositol 3,4,5-triphosphate
- PIR paired Ig-like receptor
- PLC phospholipase C
- PNGase peptide:N-glycosidase
- RBL rat basophilic leukemia
- RNA ribonucleic acid
- rVV recombinant vaccinia virus
- SAP SLAM associated protein
- SDS sodium dodecyl sulfide
- SH2 Src-homology 2
- SH2D SH2 domain protein
- SHP SH2-domain-containing protein tyrosine phosphatase
- SHIP SH2-domain-containing phosphatidylinositol 5-phosphatase
- Siglec sialic acid Ig-like lectin
- SFK Src family kinase
- SLAM signaling lymphocytic activation molecule
- SLP76 SH2 domain containing leukocyte protein of 76kDa
- TBS tris buffered saline
- TCR T-cell receptor
- TM transmembrane
- TK- thymidine kinase negative
- TREM triggering receptor expressed on myeloid
- WB Western blot
- ZAP zeta associated protein

CHAPTER I

INTRODUCTION

1.1 Introduction

Immune cell effector responses are largely controlled by a balance of signals mediated by subsets of surface proteins termed immunoregulatory receptors. Generally, these receptor-types are classified as stimulatory or inhibitory based on the intracellular signaling pathways they engage. A fine balance of signals from these receptors is required to either 'turn on' immune cells and initiate immune responses against invading pathogens (e.g. viruses, bacteria, fungi, and parasites) or 'turn off' immune responses when effector responses are no longer required . The critical importance of this system is evident since dysfunctions in immunoregulatory receptors can result in an inability to clear infections and/or the promotion of autoimmune disorders [1]. Stimulatory and inhibitory immunoregulatory receptor-types share a similar extracellular region (EC), which is responsible for ligand recognition, but differ in their transmembrane regions (TM) and cytoplasmic tails (CYT). Most stimulatory immunoregulatory receptor-types encode a positively charged amino acid (aa) residue within their TM segments and have short CYT regions that are devoid of any signaling/recruitment motifs. However, their positively-charged TM facilitates associations with several negatively-charged TM anchored adaptor molecules that have long CYT regions containing tyrosine residues embedded in immunoreceptor tyrosine-based activation motifs (ITAM). The ITAM is an amino acid motif commonly found in the CYT of TM bound proteins and is

phosphorylated at its tyrosine residues, which leads to recruitment and activation of Src family kinases (SFKs) thus initiating various signaling pathways and effector responses. In contrast, inhibitory immunoregulatory receptor-types have uncharged TM segments but encode long CYT regions, which often contain multiple tyrosine residues found within an immunoreceptor tyrosine-based inhibitory motif (ITIM). Similar to the ITAM, the ITIM is phosphorylated at tyrosine residues by SFKs leading to recruitment and activation of inhibitory tyrosine phosphatases such as Src homology 2 (SH2) domain containing tyrosine phosphatases, which dephosphorylate tyrosines embedded in signaling components involved in the effector response thus shutting down the effector response.

Most of the research characterizing these receptor systems has been performed using mammalian models (e.g. humans and mice) but the rapid control of immune effector regulation by paired receptor signaling is a theme that is constant in all vertebrates. My PhD thesis work focuses on understanding the inhibitory signaling potential of ITIM-bearing members of the leukocyte immune-type receptor (IpLITR) family in the channel catfish (*Ictalurus punctatus*). Channel catfish LITRs are a polygenic and polymorphic family of proteins belonging to the immunoglobulin superfamily (IgSF) and share features with other immunoregulatory receptors classified as stimulatory and inhibitory receptors [2, 3]. In addition, putative stimulatory and inhibitory IpLITR-types are co-expressed by various myeloid and lymphoid catfish immune cells and are thus predicted to play a key role in the regulation of different immune cell effector responses [2, 3]. The central focus of my thesis work was to investigate the signaling potential of two putative inhibitory IpLITR-types termed

IpLITR1.1b and IpLITR1.2a. My results demonstrate that these proteins recruit intracellular phosphatases via their ITIMs, which is a common strategy described for other vertebrate inhibitory receptors. In addition, I demonstrate that IpLITR recruitment of SH2-containing protein tyrosine phosphatase-1 and -2 (SHP-1 and SHP-2) was associated with their ability to abrogate immune cell effector responses (i.e. NK cell killing). Subsequent to the characterization of this well-known phosphatase-dependent inhibitory mechanism, I also characterized a new tyrosine-containing motif within the CYT region of IpLITR1.1b that is responsible for a SHP-1 and -2-independent inhibitory response. This motif recruited the C-terminal Src kinase (Csk) and this regulatory kinase was also responsible for IpLITR-mediated inhibition of NK cell killing responses.

Overall, this represents the first functional information for inhibitory IpLITR-types and reveals that catfish LITRs engage SHP-dependent and –independent inhibitory signaling pathways. Identification of phosphatase- and kinase-dependent inhibitory pathways engaged by IpLITRs is unique and sets the stage for exploring the relevance of SHP-1-dependent and –independent inhibitory signaling pathways in teleost immunity.

1.2 Objectives of this Thesis

The main objective of my research was to characterize inhibitory IpLITR-types. The specific aims of my research were: (1) To biochemically establish the inhibitory signaling potentials of two putative inhibitory IpLITRs termed IpLITR1.1b and IpLITR1.2a; and (2) To functionally establish the inhibitory role of IpLITR1.1b and IpLITR1.2a by examining their potential to inhibit cytotoxicity in mouse NK cells.

1.3 Outline and Overview

In Chapter I, I outline the objectives and the organization of my thesis. In Chapter II, I review the current literature pertaining to the role of immunoregulatory receptors in innate cellular responses. Specific emphasis is placed on reviewing the recent identification and characterization of teleost immunoregulatory receptors belonging to the Immunoglobulin Superfamily (IgSF). Chapter III contains a detailed description of the materials and methods used throughout this thesis. In Chapter IV, I describe the phosphatase recruitment potential of IpLITR1.1b and IpLITR1.2a. In order to accomplish this, I generated chimeric expression constructs in which I fused the EC and TM regions of the human inhibitory receptor, KIR2DL3, to the CYT region of IpLITR1.2a and IpLITR1.1b. Following phosphorylation of tyrosine residues in the CYT regions, I demonstrated that teleost SHP-1 and SHP-2 are recruited to these receptors thus providing strong support that ITIM-bearing IpLITRs are indeed functional inhibitory receptors. In Chapter V, I discuss the establishment of a vaccinia virus-based expression technique that was utilized to achieve expression of the chimeric KIR/IpLITR receptors on the surface of mouse splenic NK cells. In this chapter I also demonstrate that the CYT regions of IpLITR1.2a and IpLITR1.1b mediate inhibition of NK cell killing responses. Using a dominant negative recombinant SHP-1 protein, I then demonstrated that the TM proximal CYT region of IpLITR1.1b contains a specific tyrosine residue embedded in a non-ITIM motif that inhibits LAK cell killing via a SHP-1 independent process. In Chapter VI, I further investigated alternative signaling molecules that may be involved in this SHP-1-independent pathway and subsequently identified Csk as a candidate molecule. Then using co-immunoprecipitations and site-directed mutagenesis, I

confirmed that Csk was recruited to a non-ITIM motif within the TM proximal region of IpLITR1.1b and identified the tyrosine residue that was required for Csk-mediated inhibition. I also developed synthetic phosphopeptides and demonstrated that SHP-1 recruitment occurs with a canonical IpLITR ITIM while recruitment of Csk is accomplished by both canonical and non-canonical ITIM peptides. This work sets the stage for further identification of the immune effector signaling molecules that are utilized by teleost inhibitory receptors. Finally, in Chapter VII, I provide an overview of my findings and describe how these findings relate to the current understanding of immune inhibitory signaling in teleosts, and suggest future directions for the described research. Chapter VIII lists the references for the work cited in this thesis.

CHAPTER II

LITERATURE REVIEW

Portions of this chapter have been previously published:
Montgomery BC, Cortes HD, Mewes-Ares, J, Verheijen K, and Stafford JL. (2011). Teleost IgSF immunoregulatory receptors. *Developmental and Comparative Immunology* 35(12):1223-37.

2.1. Overview

Comparative immunology is a field of research that focuses on understanding the immune mechanisms of different animal species, which often involves comparing immunity in evolutionary older extant animals (i.e. non-mammals) to those of more traditional models of immunity such as humans and rodents (i.e. mammals). A significant branch of this research focuses on the immune responses of bony fishes (teleosts). Teleosts represent one of the largest classes of vertebrates that have both functional innate and adaptive immune components similar to those of mammals, thus allowing for assessments of various aspects of conserved and diverged mechanisms of immunity among vertebrates. To date, the majority of studies of teleost immune responses have focused on the identification of immune genes and immune gene families but relatively little is known on the functional significance of these molecules. This is particularly true for the large array of recently identified teleost IgSF immunoregulatory receptors. This thesis represents the work that I have done in an attempt to characterize the functional properties of a family of teleost immunoregulatory receptors called channel catfish

(*Ictalurus punctatus*) leukocyte immune-type receptors (IpLITRs). In this chapter I discuss the general characteristics of IgSF immunoregulatory receptors by giving a brief overview of some key innate immune receptor families in mammals. Following that, I discuss what is currently known about related IgSF immunoregulatory receptor families described in non-mammalian models with a strong emphasis on teleosts.

2.2 Mammalian IgSF Immunoregulatory Receptors

2.2.1 Introduction

Leukocyte responses are in part mediated by signals originating from cell surface receptors that recognize specific ligands such as pathogen-derived products. Many of these receptors are germline encoded and are generally classified as innate immune receptors. Throughout this thesis, these types of receptors will be termed immunoregulatory receptors. Many immunoregulatory receptors are transmembrane (TM) proteins consisting of an extracellular (EC) region that facilitates ligand binding, a hydrophobic TM region for anchorage in the plasma membrane, and a variable length cytoplasmic tail (CYT) that often contains multiple tyrosine residues. While some innate immunoregulatory receptor-types are found inside cells (i.e. in the cytoplasm and intracellular compartments), this review will only focus on receptor-types that are found on the cell surface. Based on their CYT region sequences, immunoregulatory receptors are also classified as either stimulatory or inhibitory due to their ability to transmit intracellular signals culminating in the promotion or abrogation of immune cell effector responses, respectively. In addition, based on the amino acid sequences of their EC regions and how these are subsequently folded, immunoregulatory receptors are grouped

into protein families, two of the major ones being the C-type lectin superfamily and the IgSF. This review chapter will focus only on IgSF immunoregulatory receptors.

Immunoregulatory receptors that belong to the IgSF family must contain one or more immunoglobulin (Ig)-like EC domain(s) with the characteristic 7-9 β-strands arranged in two β-sheets [4]. The domains in Ig and Ig-like molecules are grouped into four types: variable (V)-set, constant 1 (C1)-set, constant 2 (C2)-set, and intermediate (I)-set. These domains share a common core but differ in the number of strands in their β-sheets as well as in their overall amino acid sequences. V-set domains resemble the antibody variable domain and are found in diverse protein families including the T-cell receptor (TCR), B-cell receptor (BCR)/immunoglobulin (Ig), and several lymphocyte receptors including cluster of differentiation (CD)2, CD4, and programmed cell death protein 1 (PD-1) [5]. C1-set Ig domains resemble the antibody constant domain and are found almost exclusively in molecules that function in immune surveillance including Ig light and heavy chains and major histocompatibility complex class I and class II (MHC-I and MHC-II) [6, 7]. C2-set Ig domains are found in receptors involved in adhesion and tight conjugate formation such as CD4, vascular cell adhesion molecule (VCAM)-1, and CD2 [4]. I-set Ig domains are also found in several cell adhesion molecules including intercellular (ICAM), neural (NCAM), and mucosal addressin (MADCAM) [8]. The high diversity of Ig-like domains found in immune receptors is coupled with the vast array of ligands they recognize including microbial and viral antigens, immune proteins (e.g. antibodies, complement, and adhesion molecules), and/or altered/damaged host molecules (i.e. expressed by virus-infected and cancerous cells) [9-17]. In contrast to the high degree of specific antigen binding receptors generated through the molecular

rearrangement mechanisms of Ig and TCR genes, non-TCR and non-BCR immunoregulatory receptors are germline encoded and non-rearranging, which limits the potential number of ligands they recognize. In many cases germline encoded IgSF immunoregulatory receptors exist as large gene families consisting of closely related but polymorphic members exhibiting significant intra- and inter-species diversity [18-21]. This diversity is likely due to evolutionary pressures to match rapidly evolving ligands and/or their direct interactions with a diverse range of pathogen products [22-25]. Both myeloid and lymphoid cell types express immunoregulatory receptors including macrophages and natural killer (NK) cells. In general, the effector responses of these cells are tightly regulated by a balance of counteracting signals initiated by co-expressed receptors, which are coupled with distinct intracellular signaling modules [25-30], which will be described further below.

Paired expression of stimulatory and inhibitory immunoregulatory receptor-types form elaborate networks capable of augmenting or abrogating key immunological responses [21, 25]. For example, receptors that bind to the Fc portion of Ig (FcRs) are encoded as a cluster of genes on human chromosome (Chr) 1, which mediate macrophage and NK cell effector functions such as phagocytosis and antibody-dependent cellular cytotoxicity (ADCC) [29, 31-35]. IgSF immunoregulatory receptors that interact with MHC-I, such as killer cell Ig-like receptors (KIRs) and leukocyte Ig-like receptors (LILRs), are clustered in a region referred to as the leukocyte receptor complex (LRC) on human Chr19 and regulate NK cell cytotoxicity and cytokine secretion [19, 20, 25, 27]. In this section I will address the common characteristics of stimulatory and inhibitory immunoregulatory receptors, outline how they function, and provide some examples of

receptor families that include both stimulatory and inhibitory forms.

2.2.2 Stimulatory immunoregulatory receptor-types.

Stimulatory immunoregulatory receptors possess short CYT regions that do not contain tyrosine residues or any sequence motifs typically involved in the activation of signaling cascades. Instead, these receptors have a positively charged residue in their TM domain that facilitates an association with oppositely charged residues in the TM regions of adaptor molecules such as DAP10, DAP12, CD3ζ, and FcεRIγ, which are responsible for transducing activation signals (see Figure 2.1 for a schematic; [36, 37]). Importantly, these adaptor proteins contain immunoreceptor tyrosine-based activation motifs (ITAMs; the consensus ITAM is $YxxLx_{(7\text{-}12)}YxxL$, where x represents any amino acid) or a YxxM motif, for DAP10 [38, 39].

Depending on the immunoregulatory receptors involved, activation of immune cell effector responses occurs following recognition of specific ligands present on the surface of a pathogen or host cell, which leads to phosphorylation of the CYT regions of the associated adaptor proteins by src-family tyrosine kinases (SFKs) such as Lck or Syk. These kinases specifically phosphorylate tyrosine residues contained within the CYT ITAMs of FcεRIγ or CD3ζ [4, 40-43]. Once the ITAMs are phosphorylated, there is recruitment and phosphorylation of a number of key signaling molecules, including zeta associated protein (ZAP)-70, phospholipase C (PLC), Grb2, Vav1, phosphatidylinositol 3-kinase (PI3K), and various mitogen associated protein kinases (MAPKs) [44-46]. These activation signals lead to effector functions including tighter conjugate formation with target cells, cytolysis, cytokine release, and proliferation, and phagocytosis [44, 47-

49]. A schematic diagram of a representative effector pathway utilized by stimulatory receptors is illustrated in Figure 2.2.

2.2.3 Inhibitory immunoregulatory receptor-types

The hallmark of inhibitory immunoregulatory receptors is the possession of at least one ITIM sequence in their CYT region. The consensus ITIM sequence is S/I/L/VxYxxI/V/L [50-53] (Fig. 2.1). When inhibitory receptors are engaged by their ligands the tyrosine residues present within the ITIM are rapidly phosphorylated by SFKs [43]. Following tyrosine phosphorylation SHP-1, SHP-2, or the SH2 domain containing inositol-5 phosphatase (SHIP) are then recruited to the ITIM and subsequently activated [53-56]. The critical importance of inhibitory phosphatases in immunoregulatory receptor-mediated inhibition are observed by the immune-related diseases of motheaten mice, which are defective in SHP-1 [57, 58]. These mice display a phenotype that is characterized by focal abscesses in the skin leading to alopecia and mice homozygous for this condition have extremely low survival rate [59]. In addition, these mice display defects in NK cell function [60, 61], macrophage/monocyte function [62], and the development of T-cells [63] and B-cells [64]. Furthermore, NK cells transfected with dominant negative (DN)-SHP-1 mutants are unable to transmit negative signals via their inhibitory receptors [50, 65]. Recruitment of SHP-1 to an ITIM relieves the steric inhibition of the catalytic site and stimulates the catalytic activity of the phosphatase towards its targets, a variety of phosphorylated intermediates in the activation cascade [66]. Depending on the particular activating and inhibitory receptors engaged, recruitment and activation of SHP-1, SHP-2, and/or SHIP by the inhibitory receptors results in decreased Lck [67, 68], Lyn [69], Syk [65], PLCγ [70], ZAP-70 [71], SH2

domain containing leukocyte protein of 76 kiloDaltons (kDa) (SLP76) [72], Vav-1 [73], and PI3K [74] activities. A schematic of inhibitory phosphatases and some of their identified targets are illustrated in Figure 2.3.

2.3. IgSF immunoregulatory receptors and NK cells

The host's ability to detect and remove infected cells, transformed cells, and cells of foreign origin (i.e. foreign tissue transplants) has been in large part attributed to the function of NK cells, which are large granular lymphocytes that play an essential role in innate immunity [75]. Functionally, NK cells produce cytokines, especially interferon (IFN)-γ and tumour necrosis factor (TNF)-α, and/or lyse target cells [75, 76]. NK cells have the ability to directly kill altered self-cells, such as virally infected cells or transformed (i.e. cancerous) cells by releasing the contents of pre-formed cytotoxic granules upon target cell contact [77]. NK cells can also mediate ADCC via surface FcRs binding to the Fc portion of the IgG antibody on an opsonized target [78].

NK cells have the innate potential to kill and this must be tightly regulated to prevent injury to healthy tissue. To control their functions, NK cells express a complex array of receptors that discriminate infected from uninfected cells (e.g. tumor cells from healthy cells or infected cells from uninfected cells). Some of these receptors recognize MHC-I as ligands on target cells and regulate the functional response of the NK cell to the target cell, either positively towards activation or negatively towards inhibition [30, 78-80]. IgSF immunoregulatory receptors such as the KIRs, LILRs, and FcRs are known to have an important role in the regulation of NK cell-mediated innate self/non-self differentiation as described further below.

2.3.1. KIRs

The 150 kb KIR locus is present in the leukocyte receptor complex (LRC) on chromosome 19p13.4. The LRC is an extended region encoding a large selection of IgSF receptors interspersed with other loci [25]. KIRs are expressed on subsets of NK cells and a subset of T cells. The basic structure of KIR is 2 or 3 EC Ig-like domains termed KIR2D or KIR3D, respectively. These extracellular Ig domains specifically recognize and bind to human leukocyte antigen (HLA) class I molecules [81] and there are more recent reports of diverse ligands such as CpG DNA [82]. KIRs are further classified according to the length of their CYTs as either long or short, which possess different functional properties. Inhibitory KIRs, termed KIR2DL and KIR3DL, possess a long CYT tails that encode one or more ITIMs, which mediate inhibitory signaling [23]. Stimulatory KIRs possess short CYT tails and are termed KIR2DS and KIR3DS. The KIR2DS receptors are associated with activating signals due to the adaptor ITAM-bearing adaptor, DAP12 [46]. Individual KIRs recognize a limited repertoire of MHC-I alleles [83]. There is not a KIR for each specific MHC-I molecule but instead each KIR recognizes determinants that are shared by a group of MHC-I molecules. Specifically, KIRs bind to polymorphic determinants in the α1 domain of human leukocyte antigen (HLA)-A, -B, and -C molecules [84].

2.3.1.1. Inhibitory KIRs

NK cell cytotoxicity is triggered by the engagement of various activating receptors expressed by NK cells when these interact with target cells. Negative regulation of NK cell cytotoxicity induced by the recognition of MHC-I molecules on

target cells by inhibitory KIRs is an efficient means to protect healthy autologous cells from death by NK cells. Infected or transformed cells often display a reduced expression of MHC-I, which enables them to escape death from conventional cytotoxic T cells (CTLs). HLA-C is a particularly specialized human MHC-I molecule towards control of the NK cell response since all allotypes of HLA-C are KIR ligands [85]. HLA-C molecules can be grouped into two subsets based on the amino acid residues 77 and 80 in the α1 helix [86]. Group I HLA-C epitopes are characterized by a serine at position 77 and an asparagine at position 80. These include the HLA-Cw allotypes 1,3,7,8,13,14 and are recognized by KIR2DL2/3 [85]. Group 2 HLA-C epitopes are characterized by an asparagine at 77 and lysine at 80, these include HLA-Cw2,4,5,6,15,17, and 18 and are recognized by KIR2DL1[85]. Inhibitory KIR3D have 3 Ig-like domains, D0, D1, and D2 [87]. KIR3DL1 recognizes epitopes of HLA-Bw4 while KIR3DL2 binds to epitopes shared by HLA-A3 and HLA-A11 [88, 89]. There are still many alleles of HLA-A and HLA-B for which a corresponding KIR is yet to be identified and may mean that this role is played by other receptor families. The inhibitory KIR receptors were the first MHC-I receptors to be found to contain CYT ITIMs as well as the first receptors that were discovered to inhibit NK effector responses through the recruitment of SHP-1 and SHP-2 but not SHIP [50]. Following these discoveries, the search for inhibitory immunoregulatory receptors has led to the discovery of many diverse receptor families and new understandings into self vs. non-self recognition.

2.3.1.2. Stimulatory KIRs

Certain pairs of KIRs have similar extracellular domains but differ in their signaling function (i.e. the TM and CYT regions). KIR2DL1 and KIR2DL2/3 pair with

the stimulatory receptors KIR2DS1 and KIR2DS2 [85, 90]. Similarly, KIR3DL1 is paired with the KIR3DS1 [91]. These relationships suggest that KIR signaling function might have switched from inhibitory to stimulatory or vice versa during evolution [92]. As described in section 2.3.1.1., the ligand binding specificities of the inhibitory KIRs are well characterized while there is relatively little information regarding the ligand-binding abilities of the stimulatory forms. There has been evidence to suggest that short-tailed KIRs bind with lower affinity to HLA-C than inhibitory KIRs [81] while other binding studies have shown that the KIR2DS receptors do not bind to HLA-C at all [93, 94]. During HIV infection, patients expressing both KIR3DS1 and certain allotypes of HLA-B were found to have much slower acquired immune deficiency syndrome (AIDS) progression [95]. Stimulatory KIRs, specifically KIR2DS1 and KIR2DS2, have also been implicated in the development of autoimmune disorders such as psoriatic arthritis and type I diabetes [96, 97]. At this time further examination is required to determine this relationship and additional ligands remain unknown for KIR2DS2 and KIR3DS1 [98]. In both NK cells and subsets of T cells, stimulatory KIRs initiate effector responses through signals mediated from an associated DAP12 molecule [99, 100].

2.3.1.3. KIR2DL4 displays characteristics of both stimulatory and inhibitory immunoregulatory receptors

KIR2DL4 is unique in many regards when compared to other KIRs and other IgSF immunoregulatory receptors in general. It is the only KIR that is ubiquitously expressed on all NK cells and a subset of T-cells [101, 102]. It is also unique in that it localizes to endosomes while other KIRs localize to the cell surface [103]. The only known ligand for KIR2DL4 is HLA-G, a non-classical MHC-I molecule that is restricted to expression in fetal trophoblast cells that invade the maternal decidua during early

pregnancy [104]. At this site, KIR2DL4 signals to induce a proangiogenic and proinflammatory response through the production and release of cytokines and chemokines. The importance of this interaction is reinforced as the loss of HLA-G can lead to the spontaneous abortion of the fetus [105]. KIR2DL4 possesses the potential to act as a stimulatory or inhibitory receptor as it contains a long CYT containing one ITIM in addition to recruiting the ITAM-bearing adaptor molecule FcRγ [101, 106]. The CYT of KIR2DL4 has been shown to recruit SHP-2 to the ITIM region but not SHP-1 and mutation of the tyrosine residue within the ITIM did not completely abolish the inhibitory signaling potential [107]. Recently, it was reported by Miah *et al*., that the CYT region is ubiquitylated by Triad3A, which leads to its degradation [108]. This may suggest that the CYT region also plays a role in the regulation of the KIR2DL4 signaling, albeit not in the way that is commonly proposed for inhibitory receptors.

2.3.2. LILRs and related IgSF families

The leukocyte Ig-like receptor (LILR) family is another IgSF family located in the leukocyte receptor complex (LRC) region. The LILR family is encoded by 13 genes at two loci within the LRC on 19q13.4 [109]. Within the LRC, the KIR gene cluster is found immediately telomeric of the LILR genes [110]. The two LILR loci are separated by a region of ~200 Kb that is flanked by 2 genes from a separate receptor family termed leukocyte–associated Ig-like receptors (LAIRs) [109, 111, 112]. In contrast to the KIRs, the LILR introns and intergenic regions are not highly homologous and LILR loci are probably older than the KIR genes [20]. There are 13 members of the LILR family, and they can be classified as having the characteristics of either inhibitory immunoregulatory receptors, with an uncharged TM region and ITIM-bearing CYT tail, or stimulatory, with

an arginine residue located in the TM region and a short CYT tail [113]. These proteins are expressed on a wide range of immune cells including B-cells, T-cells, dendritic cells, monocytes, mast cells, and NK cells [114].

2.3.2.1. Inhibitory LILRs

To date, most information about the LILR comes from the inhibitory LILR forms, LILRB1 and LILRB2. The first LILRB1 ligand described was the HCMV UL18 gene product, an MHC homolog [9, 115]. These findings were some of the first evidence that viruses have evolved mechanisms to mimic host self-ligands as a means of protection from the host immune response. Although the extracellular (EC) region of LILRB2 shares high homology with LILRB1, it binds to UL18 with ~1500 fold less affinity. In addition, LILRB1and LILRB2 both recognize alleles of HLA-A, HLA-B, and HLA-C as cognate ligands [27]. Non-classical ligands include UL18, an HCMV protein that resembles MHC-I was found to bind LILRB1 [9]. ITIMs in the CYT inhibit cellular responses by recruiting SHP-1 [114].

2.3.2.2. Stimulatory LILRs

Relative to the inhibitory LILR types, there is much less information about the characteristics of the stimulatory LILR types. LILRA2 has been implicated in the activation of eosinophils [116] and antibody (Ab) cross-linking of ILT11/LIR9 results in the secretion of proinflammatory cytokines such as interleukin (IL)-1β, TNF-α, and IL-6 [117]. It has been demonstrated that these stimulatory signals are initiated through LILR association with the ITAM-bearing adaptor FcεRIγ [118].

2.3.2.3. Paired Ig-like receptors (PIRs)

The further examination of paired IgSF families in vertebrates has revealed an orthologous relationship to receptor families in rodents, the paired Ig-like receptors (PIRs). The PIR receptor family was first identified in mice as a receptor family homologous to FcαR1, the human receptor for IgA [119, 120]. Searches of the mouse genome revealed six stimulatory PIR forms, termed PIR-A, and one inhibitory PIR form, termed PIR-B in a chromosomal region syntenic to the human LRC [121, 122]. Initial studies did not observe any PIR binding to IgA or other immunoglobulin isotypes and the focus shifted towards a proposal that PIRs are orthologues of the LILR family due to similarities in structure, expression profile, and genomic localization [19, 123, 124]. Reinforcing the idea of an orthologous relationship between PIRs and LILRs, it was demonstrated by multiple groups that PIRs engage both classical and non-classical MHC-I in a manner similar to that of LILR recognition of human MHC-I [123-126].

2.3.2.4. Chicken Ig-like receptors (CHIRs)

The first identification of the CHIR family was established with the characterization of two chicken receptors with homology to the mouse PIR genes [127]. From several chicken EST databases, multiple CHIR members were discovered and designated as CHIR-A for the activating types and CHIR-B for the inhibitory types [127, 128]. Further investigation provided evidence to suggest that the CHIR locus expanded to include over 100 CHIR genes and was located on microchromosome 31, which represents the orthologous region of the mammalian LRC [129]. Additionally, the CHIRs show equal homology to both the KIR and LILR in humans and in some cases

combine features of both, making it difficult to conclude at this time whether the CHIRs are ancestral to the KIR and/or LILR gene families [130].

All CHIRs have one or two Ig domains in the EC region and these Ig domains can be further classified into the C2 type, similar to the Ig domains of KIRs and LILRs [129]. CHIRs can be divided into three general subtypes depending on the TM and CYT regions: CHIR-B, CHIR-A, and CHIR-AB [129]. Similar to traditional immunoregulatory inhibitory receptors, CHIR-B have a TM region lacking any charged residues and a long CYT tail either two ITIMS or a membrane distal ITIM and an ITSM. CHIR-B ligation leads to the recruitment of SHP-1 and SHP-2 resulting in the inhibition of proliferation in a chicken B cell line [128]. CHIR-A members feature one or two Ig domains and a basic residue in the TM region [131]. Cotransfection studies indicate that FcεRIγ, but not CD3ζ associates with CHIR-A [129]. The CHIR-AB subgroup combines features of CHIR-A and CHIR-B in that it contains a long cytoplasmic region with signaling motifs and a basic residue with the TM region. These traits suggest that CHIR-AB may have the potential to initiate opposite functional outcomes and this may be dependent on different factors including: availability of adaptor molecules, cell type, or availability of appropriate ligands [130]. Through the use of a BWZ reporter cell assay, it was discovered that CHIR-AB1 recognized chicken IgY, an ancestral form of both the mammalian IgG and IgE isotypes [132]. The discovery and characterization of the CHIR family has lead to a significant increase in knowledge regarding the evolution of LRC receptor families and further examination will continue to expand our understanding into the function and organization of homologous immunoregulatory receptor families.

2.3.3. FcRs

Several subsets of immune cells are armed with integral membrane receptors specific for the Fc portion of soluble Ig, i.e. FcR. Depending on the type of FcR engaged and/or the immune cell-type involved, these proteins participate in a range of immune responses including the uptake and clearance of immune complexes, phagocytosis of pathogens, secretion of cytokines/chemokines and ADCC [29, 32-34, 49]. FcγRIII, or CD16, is a TM-anchored glycoprotein of the IgSF expressed on the majority of human NK cells [43]. CD16 is a low-affinity receptor for IgG and ligation of CD16 with, for example, opsonized target cells initiates NK-mediated cytokine production and ADCC [34]. CD16 is non-covalently associated with subunits containing an ITAM, such as the γ-subunit of the high affinity receptor FcεRIγ or the ζ subunit of the TCR complex of human NK cells [43, 133]. The signal transduction pathways and the effector functions induced by activation of CD16 on NK cells are remarkably similar to the events triggered by engagement of the TCR on T-cells. CD32, or FcγRIIb is a low affinity IgG receptor that differs from CD16 in that it does not associate with ITAM bearing adaptor molecules and encodes an ITIM in its longer CYT. This ITIM has been demonstrated to recruit SHIP in both B cells and mast cells and this leads to inhibition of calcium influx and downstream responses [134].

2.3.4. Other mammalian IgSF immunoregulatory receptors.

The central importance of FcR-, KIR-, or LILR-mediated regulation of cellular effector responses is highlighted by studies implicating receptor dysfunctions with

increased susceptibility to certain diseases. Thus, dysregulated immune cell responses traced to aberrant FcR, KIR, and LILR functions can have destructive biological consequences resulting in autoimmunity, malignancy, and infections [1, 27, 29, 135]. While the aforementioned proteins exhibit key immunoregulatory roles, they are not the only receptor-types known to participate in shaping mammalian immune cell responses. For example, a series of other IgSF immunoregulatory members that serve as co-receptors (e.g.B7 and CD28/CTLA4), adhesion molecules (e.g. CD2 and carcinoembryonic antigen-related cell adhesion molecule [CEACAM]), triggering receptors expressed on myeloid cells (TREMs), natural cytotoxicity receptors (NCRs; e.g. NKp30, NKp44, and NKp46), and sialic acid-recognizing IgSF lectins (Siglecs) are also present on immune cell subsets and have been demonstrated to elicit profound immunomodulatory effects [17, 136-141]. In addition, many non-IgSF receptors such as CD94/NKG2 and Ly49 C-type lectin family members can influence key immunological responses in mammals [142], reinforcing that a complex array of receptor types participate in dictating innate immune cell functions.

2.4. IgSF Immunoregulatory Receptors in Non-Mammalian Animals

2.4.1. Introduction

Repertoires of immunoregulatory receptors have been discovered in non-mammalian vertebrates (i.e. birds, amphibians, and fish) and many of these belong to the IgSF [2, 127, 130, 137, 143-153]. This further emphasizes the complexity of vertebrate immune system regulation and provides insights into the evolutionary origins of vertebrate immunoregulatory receptor networks. Examination of the signaling pathways initiated by non-mammalian immunoregulatory receptors has also shown that a diverse

range of receptor-types engage (or are predicted to engage) evolutionarily conserved signaling pathways required for the initiation and termination of immune responses [128, 145, 154-157]. Although Mmuch has been learned in recent years from studies in amphibian and avian species, most notably the recent discovery and characterization of the chicken Ig-like receptor (CHIR) family, which I will cover briefly. , With the exception of the CHIR family, tthis next section will focus on IgSF immunoregulatory receptors that have been described in fish.

2.4.2. IgSF Immunoregulatory receptors in fish

Over the past two decades, comparative immunologists have collectively contributed to the cloning and molecular characterization of an assortment of immune-related genes from several fish species, which has significantly advanced our understanding of teleost immunity [151, 158, 159]. In combination with this vast molecular inventory, the establishment of procedures for the isolation, separation, and long-term in vitro cultivation of fish leukocytes has been instrumental towards the functional characterization of cellular subpopulations and cellular immune responses in teleosts [160-170]. For example, it is well documented that teleost immune cells are capable of phagocytosis (macrophages and B cells) [171-174], cellular cytotoxicity (NK-like cells and cytotoxic T lymphocytes) [169, 170, 175, 176], ADCC (NK-like cells) [170, 177], antigen presentation (macrophages) [178, 179], and degranulation (mast cells and neutrophils) [180-182]. Although these functional responses are highly reminiscent of the responses elicited by mammalian immune cells, the immunoregulatory receptors responsible for controlling and coordinating these cellular events remain unknown.

Questions such as “Are phagocytic and ADCC processes mediated by teleost FcR-like proteins?” and “Are teleost NK cell cytotoxic events regulated by receptor networks reminiscent of NCR, KIR- and/or LILR-like proteins?” cannot be convincingly answered at present. The discovery of novel immune-type receptors (NITRs; reviewed in [183] and discussed further below) suggests that teleost innate immune responses may be in part regulated by specialized and unique receptor networks exclusive to fish. However, additional IgSF members from several fish species have recently been identified providing important new insights into the molecular and functional evolution of IgSF immunoregulatory networks [2, 149, 184]. This section will give an overview of the most recently discovered teleost innate immune receptors including: NITRs, FcRs, novel Ig-like transcripts (NILTs), modular domain immune-type receptors (MDIRs), diversified intermediate domain-containing proteins (DICPs), and the leukocyte immune-type receptors (LITRs). Less focus will be spent on the NITR family of receptors as it is one of the original teleost IgSF immunoregulatory receptor families to be identified and characterized. Many excellent reviews are available for NITRs [183, 185-187]. This portion of the review will focus on the more recently discovered teleost immunoregulatory receptors.

2.4.3. Teleost NITRs

The first teleost NITR was cloned from the Southern pufferfish (*Spheroides nephelus*) in a search to identify V-set genes that do not undergo V(D)J recombination (i.e. unlike Ig or the TCR) [188]. This prototypical NITR, termed ‘Sn193’, encodes a type I TM receptor containing an extracellular V-type and I-type Ig domain and a TM region connected to a CYT containing both ITIM and ITIM-like sequences. Subsequent

studies revealed additional NITR genes and/or cDNAs in Southern pufferfish [146], zebrafish (*Danio rerio*) [147, 189], rainbow trout (*Oncorhynchus mykiss*) [190], channel catfish (*Ictalurus punctatus*) [191, 192], Japanese flounder (*Paralichthys olivaceus*) [193], medaka (*Oryzias latipes*) [194], Fugu (*Takifugu rubripes*) [148], *Tetraodon nigroviridis [194]*, and European sea bass (*Dicentrarchus labrax*) [195]. The NITR gene clusters have been fully annotated for zebrafish and Japanese medaka, which has exposed the large gene content of this receptor family [148, 194].

2.4.3.1. NITR sequence analysis and expression

Thirty-six zebrafish NITR genes representing 12 families of NITRs have been identified from the zebrafish genome in a single gene cluster on chromosome 7 [148]. Additionally, three more NITR genes are encoded in a gene cluster on zebrafish chromosome 14 [189]. All zebrafish NITRs are expressed in the kidney, spleen, and intestine as detected by RT-PCR but only *nitr3* was expressed during embryogenesis [196]. Leukocytes of both lymphoid and myeloid lineage were measured for NITR expression with only the lymphoid population testing positive [196] although there is evidence that, in channel catfish, NITRs are expressed in a channel catfish macrophage-like cell line [191].

2.4.3.2 *Danio rerio* NITR9L signals through the adaptor DAP12

Zebrafish NITR9L, DrNITR9L, the long isoform of NITR9, was found to associate with DAP12 but not DAP10 when expression constructs of NITR9 and either DAP10 or DAP12 were co-transfected into the AD-293 cell line, a modified HEK 293 cell line with enhanced adhesion properties [155]. Antibody cross-linking of NITR9L

promoted its association with DAP12 that resulted in phosphorylation of ERK and AKT indicating that DrDAP12 utilizes the PI3K-ERK pathway similar to mammalian innate stimulatory receptors [155].

2.4.3.3 IpNITR11 demonstrates recognition of an allogeneic cell line

On the basis of both their domain organization and diverse V structures, it was hypothesized that NITRs mediate allogeneic recognition [147, 151]. Nine families of NITRs have been described in catfish and a number of different NITRs are expressed by 3H9, a cell line that demonstrates cytotoxicity towards allogeneic catfish B cell lines [170]. In order to examine the potential interactions of NITRs with the 1G8 and 3B11 cell lines, chimeric constructs consisting of a FLAG epitope tagged NITR extracellular domain fused to the TM of skate MDIR2 and the CYT region of mouse CD3ζ were generated. These NITR fusion constructs were then transfected into the 43-1 T cell hybridoma line, which contains a green fluorescent protein (GFP) reporter gene under the control of a nuclear factor of activated T cells (NFAT)-responsive promoter [197]. cDNA fragments encoding the extracellular domains of nine separate catfish NITRs were also transfected into the reporter cell line. Only NITR11 induced GFP expression for the 1G8 B cell line but not 3B11 indicating a specific interaction. This recognition of NITR11 to the 1G8 cell line was dependent on a single asparagine residue at position 50 and when this was mutated to aspartic acid, the binding of NITR Fc-fusion proteins to the 1G8 cell line were lost. Conversely, the reciprocal point mutation on NITR10 (D50N), which shares the most similarity with NITR11 but does recognize the 1G8 cell line, resulted in strong reactivity with the 1G8 cell line [198]. Glycine treatment, which has been shown to disrupt the interaction between MHC-I and β2M [199, 200], resulted in no

change in binding ability of NITR11 to the 1G8 cell line. Therefore, this recognition does not appear to be mediated by MHC-I, which is an important self/non-self ligand on host cells for the mammalian KIR and LILR receptor families. What these results suggest is that the allogeneic recognition response is mediated by a surface molecule, which is not affected by low pH treatment (e.g. glycine pH 2.5). Further studies have also ruled out Ig as a NITR ligand and, to date, the nature of any NITR ligands remains a mystery [198].

2.4.4. Teleost FcRs

Although several genes encoding for FcR and FcR-like (FCRL) proteins exist in mammals [49, 201], birds [127, 128, 131, 154, 202] and amphibians [144, 145, 203] very little is known about the nature of these receptors in teleost. Functional evidence suggests that fish immune cells bind Ig via putative FcRs, which significantly influences their effector responses [204-206]. One of the strongest cases for the existence of an FcR homolog in fish was derived from studies in channel catfish (*Ictalurus punctatus*) [207]. These experiments showed that catfish NK-like cells are armed with serum-derived IgM via a putative stimulatory FcμR that induced an ADCC response[207]. Subsequently, the generation of an IgD-specific monoclonal Ab (mAb) revealed that channel catfish granulocytes are armed with IgD, which induced a degranulation response following mAb- mediated cross-linking [182]. Finally, the new finding that subsets of teleost IgM^+ and IgT^+ B cells exhibit the classical myeloid cell functions of phagocytosis and intracellular killing raises the distinct possibility that these are also FcR-mediated processes [174, 208]. The discovery and characterization of fish FcRs is paramount to understanding the principal facets of teleost FcR biology.

2.4.4.1. Discovery and characterization of a channel catfish FcR

In 2006, database screens using mammalian FcR sequences as queries resulted in the identification of a putative channel catfish FcR homolog referred to as IpFcRI [209]. This gene was highly related to FcR and FCRL receptors in amphibians and endothermic vertebrates. A combination of phylogenetic analysis and structural predictions further reinforced that IpFcRI is a true teleost FcR homolog with potential Ig-binding properties [209]. IpFcRI is a 311 aa protein consisting of a 19 aa leader peptide followed by three predicted C2 Ig-like domains but is devoid of a TM segment and CYT region. The mature protein has a predicted weight of ~33 kDa and contains five N-linked glycosylation sites that may influence the overall size of the mature protein. No glycophosphatidylinositol (GPI)-anchorage sites were identified at the C-terminal region of IpFcRI, suggesting that this is a secreted FcR or perhaps it associates with the cell membrane by utilizing membrane-bound adaptor protein. IpFcRI encodes three domains, but only the membrane distal D1 and D2 share phylogenetic relationships with the corresponding domains of other FcRs [209]. Secondary structural predictions demonstrated that the β-strands in IpFcRI are present in similar positions as the β-strands found in the mammalian FcR family members with the exceptions of an additional IpFcR β-strand predicted within the D1 between the F and G strands as well as a missing C′ β-strand within the D2 [209]. Comparative homology modeling also indicates an overall conservation of IpFcRI D1D2 folding in comparison to mammalian FcRs and a putative Ig-binding site was also identified [209]. IpFcRI is a single copy gene and expressed transcripts were detected by Northern blot in the spleen, hematopoietic kidney tissues and peripheral blood leukocytes (PBL). Lower levels of expression were observed in the

heart, gill, and liver as well as the majority of clonal leukocyte cell lines examined including macrophages and B cells. Quantitative PCR analysis using PBL subpopulations indicated that circulating granulocytes predominantly expressed IpFcRI, whereas the IgM surface positive cells (B cells and/or NK-like cells) and the IgM negative lymphocytes express significantly lower levels. Conversely, IgM negative lymphocytes were the major IpFcRI expressing cells when isolated from kidney tissues. These various expression profiles suggest that cell-type and tissue location can alter IpFcRI expression in vivo [209].

Using an insect cell expression system, recombinant IpFcRI was confirmed to be a secreted glycoprotein [209]. Two immunoreactive bands were also detected in catfish plasma with approximate weights of 64 and 90 kDa using a rabbit polyclonal anti-IpFcRI Ab. These proteins are significantly larger than the predicted weight of the polypeptide (~33 kDa) but following deglycosylation by peptide-N-glycosidase (PNGase) F treatment, the 64 kDa band was reduced to 40, 50, and 55kDa, which is remarkably similar to the banding pattern observed following deglycosylation of a mammalian soluble FcR [209, 210]. The 90 kDa protein was reduced to 70 kDa after deglycosylation but the identity of this protein remains unknown. Even though IpFcRI is secreted it still maintains Ig-binding properties [209]. For example, using a combination of co-immunoprecipitation experiments and cellular transfection studies, it was demonstrated that IpFcRI directly bound serum-derived and column-purified catfish IgM indicating that IpFcRI is indeed a bona fide teleost FcμR [209]. This work represents the first description of an Ig-binding receptor discovered in an ectothermic vertebrate.

Subsequent work has focused on identification of the IpFcμR–IgM binding sites

and a linear epitope on the catfish IgM Fc region has since been identified as part of this interface [211]. The linear epitope is formed by a consensus octapeptide motif (FxCxVxHE) that was mapped using custom-made oligopeptides to the second cysteine responsible for forming the intrachain disulphide bonds for both the Cμ3 and Cμ4 within the heavy chain of catfish IgM. Conservation of this linear epitope among other vertebrate Ig molecules was observed and the ability of soluble IpFcRI to bind several antibody classes from a broad range of vertebrate taxa including rainbow trout, chicken, mouse, rabbit, and goat were reported [211]. In addition, IpFcRI bound mouse IgM, IgG, and IgA when these antibodies were immobilized onto latex beads indicating that the IpFcRI binding site is also exposed under native conditions. The original description of IpFcRI combined with recent information regarding its Ig-binding potential represents an important first look into the evolution of FcRs; however the in vivo role of soluble IpFcRI remains unknown. In addition, ongoing studies are required to fully appreciate how teleost FcRs influence immune cell responses and in particular what the identity is of the receptors leading to NK cell-mediated ADCC, macrophage and B cell phagocytosis, as well as granulocyte degranulation.

2.4.5. Teleost Modular Domain Immune-Type Receptors (MDIRs)

MDIRs represent a multigene family of activating/inhibitory receptors belonging to the IgSF originally discovered in the clearnose skate (*Raja eglanteria*) [212]. Oligonucleotides complementing sequences that included the conserved V-domain NITR gene sequences were used to prime skate spleen cDNA. From this process, five distinct, full-copy-length cDNA sequences were identified, encoding IgSF TM proteins termed MDIR1-5 [212]. Extracellular Ig domains range in number and type. For example,

MDIR1 encodes one intermediate (I)-set IgSF domain while MDIR2, 3, 4, and 5 encode for a distal I-set IgSF domain with a variable number of proximal I-set and C2-set IgSF domains, which range from two to six [212]. MDIR1 contains a basic residue within its TM region while MDIR2, 3, 4, and 5 all contain TM regions but lack any charged residues in addition to CYT tails with MDIR2 and 3 encoding ITIM and ITIM-like regions within their CYT tails [212].

Basic Local Alignment Search Tool (BLAST) database searches using full-length predicted MDIR proteins revealed orthologs in a wide range of vertebrate groups including teleosts, amphibians, birds and mammals [212]. MDIRs are most similar to the CD300 IgSF family with lesser similarity to the extracellular domains of the triggering receptor expressed on myeloid cells (TREM), TREM-like transcritpt (TLT), T-cell immunoglobulin and mucin domain (TIM), and pIgR IgSF families [212-215]. Skate MDIR and mouse CD300 domains were then used as TBLASTN queries to identify a large set of MDIR-type in the zebrafish genome. The genes reside in two major complexes, each consisting of multiple loci and further examination of these loci revealed at least 20 MDIR-type domains [212].

2.4.5.1. Identification of Skate MDIR1 ligands

Although the binding partners to zebrafish MDIRs remain unknown, skate MDIR1 has been found to bind specific polar lipids including phosphatidylcholine (PC), phosphatidylethanolamine (PE), and cardiolipin (CL) [216]. This ligand specificity is consistent with related receptors in mammals including CLM1, CLM3, CLM5, CLM7, CLM9, TREM2, TREML1, TREML2, TREML4, and TREML6 [216]. These results suggest that the strategy of immune recognition of lipids is a conserved ancient strategy

of recognition for conserved patterns displayed on potential pathogens.

2.4.6. Teleost DICPs

Ig V-, I- and C2-set motifs from NITRs and MDIRs were used as queries in tBLASTn searches of the zebrafish genome to identify unrecognized Ig-region genes. The results of this robust search yielded the DICP IgSF family [184]. The typical DICP is a type I TM receptor that consists of two distinct classes of extracellular Ig domains with the D1 domain sharing conservation with the classical V-set domain than the D2 domain [217]. Paired cysteine residues were found in both D1 and D2 domains and predicted to form intrachain disulfide bonds. Twenty-nine D1 domains were identified on zebrafish chromosomes 3, 14, and 16. The designation system of these receptor genes follows a number that indicates the gene cluster (1, 2, or 3), followed by a number that indicates the order in which the gene was identified and a superscript that indicates an allele sequence source. For example *dicp1.7*^{262} refers to first cluster, seventh D1 domain and scaffold 262. BLAST searches were used with DICP D1and D2 nucleotide sequences as queries. Although DICP-related sequences were identified in carp, tilapia, pufferfish, and salmon, only one definitive DICP transcript was identified in carp [184].

2.4.6.1 DICP transcript analysis

The sequencing of multiple DICP expressed sequence tags (ESTs) and cDNAs facilitated the characterization of the exon organization and putative translation products from a large number of highly related candidate DICP genes. Most DICP domain exons are flanked by exons that encode a leader signal sequence and a D2 domain exon although some genes are found adjacent to leader sequences but lack the D2 domain. Based on the genomic assemblies, no fewer than 27 DICP genes and pseudogenes were

identified. Important features of the DICP family uncovered from this analysis include: i) although there is high variability in the D1 and D2 domains of Dicp1.2, 1.11, 1.16, and 1.19, the TM and CYT regions of several DICP members are highly conserved (i.e. they differ by no more than one residue); ii) several DICPs lack the D2 domains; iii) Dicp1.7 and 1.8 are identical; and iv) alternative splicing produces multiple forms of Dicp1.17, Dicp2.2, and Dicp3.1. Overall, DICPs vary in terms of predicted ectodomains, presence/absence of ITIM motifs. A schematic representation of these receptors can be found in Figure 2.4.

2.4.6.2 DICPs bind lipids with variable affinity

Enzyme-linked immunosorbent assays (ELISAs) with human (h)Fc fusion proteins containing the D1 domain of various DICP ECs revealed that DICP1.5, 1.6, and 1.19 all bound cardiolipin at differing affinities [184]. DICP1.5 was also demonstrated to bind soluble extracts from four different classes of bacteria including: *Gammaproteobacteria*, *Bacilli*, *Actinobacteria*, and *Flavobacterium* [184]. This appears to be a strategy of recognition of conserved PAMPs from various bacteria and further investigation may reveal information for IgSF receptor families as an ancient co-evolved pathogen defense strategy akin to TLRs.

2.4.7. Teleost NILTs

The first reported NILT sequences were obtained in 2005 from a carp macrophage cDNA library [149]. These genes encode predicted proteins belonging to the IgSF that are distantly related to the human immune proteins CMRF-35 leukocyte receptor [218, 219], TREMs [220], and the natural cytotoxicity receptor NKp44 [220]. These NILT genes were termed Cyca-NILT1 and Cyca-NILT2 based on their predicted stimulatory

and inhibitory signaling capabilities, respectively, and in 2008 an additional NILT sequence (termed Onmy-NILTD) was discovered by database mining for homologues of activating NK cell receptors in trout [221]. Subsequently, in 2009 the identification of three additional NILT genes was reported (Onmy-NILT2, Onmy-NILT3, and Onmy-NILT4) bringing the total repertoire of teleost NILTs to six [153]. Based on genomic DNA analysis, Cyca-NILTs are members of a large family of immune genes, which was verified by the examination of cDNA clones isolated from an individual carp, leading to the identification of 53 additional NILT sequences [149]. When NILT searches were extended to six more carp, an even larger repertoire (>100 sequences) of NILTs were uncovered (i.e. 30 Cyca-NILT1-like and 95 Cyca-NILT2-like) and four of these NILTs were shown to be present in all of the individuals examined [149]. Sequence analyses identified a considerable amount of aa variability within the Ig-like domains of carp NILTs indicating that these immunoregulatory receptors are polymorphic [149]. This polymorphism is similar to that observed for teleost NITRs [146-148] and other non-rearranging innate immune receptors in vertebrates [2, 19-21, 25, 27, 127, 135, 145, 222]. Just recently, it was reported that screening of an Atlantic salmon genome library identified six novel NILT genes and confirmed the tightly clustered and multigene nature of these immunoregulatory receptors [153].

2.4.7.1. NILT sequence analysis

Mining of the zebrafish genome using Cyca-NILT1 and Cyca- NILT2 sequences identified two regions spanning 270kb on Chr 1 [149] containing a high density of NILT-related exons that were ~10 megabases (MB) upstream of the Dare-ZW loci encoding MHC-I heavy chains [223]. Similarly, mining for Onmy-NILT related sequences

identified ~50 Ig-like domain sequences on zebrafish Chr 1 with ~40% identity and two additional loci (Chr 2 and Chr 15) encoding NILT-like Ig domains (25–40% identity) [221]. Distant relatives of the teleost NILTs also exist in mammals, which provide important insights into their potential functional significance [149]. For example, mining of the human genome for Cyca-NILT relatives retrieved the highest scoring match as IREM1, which is a receptor belonging to the CMRF-35 family on Chr 17q25.1 [218, 219]. In addition, several hits on human Chr 6p21.1 in a region encoding TREM and NKp44 were also indicated as NILT relatives. Both CMRF35 and NKp44 genes are IgSF immunoregulatory receptors expressed by various immune cell types, which are found among clusters of closely related human genes [218-220, 224]. This scenario is reminiscent of the multigene properties described for NILTs (i.e. multiple related NILTs are likely clustered together in the teleost genome) and it seems plausible that NILTs are orthologous to either human gene clusters on Chr 6p21.1 and/or 17q25.1. Evidence for this potential orthologous relationship is reinforced by the fact that 10 MB upstream from the NKp44 and TREM genes on human Chr 6 are the MHC class I genes at position p21.31 [220]. This is remarkably similar to the aforementioned location of Dare-NILT homologs on zebrafish Chr 1 approximately the same distance from the MHC-I Dare-ZE genes [223].

2.4.7.2. NILT predicted protein structures

The prototypical teleost NILT structure has been gained from analysis of the predicted protein sequences and homology modeling [149]. All known NILTs have the same basic structure consisting of a leader peptide, one or more Ig-like domains, a connecting peptide, a TM segment and CYT tail (Fig. 2.5). The one major exception is

Onmy-NILT2D, which is alternatively spliced into a protein lacking the TM and CYT regions and is presumably secreted [221]. Another alternatively spliced trout NILT results in the generation of two isoforms encoding either one (Onmy-NILT4 Short) or two (Onmy-NILT4 Long) Ig-like domains due to the deletion (or addition) of its second Ig-like domain [152]. However, unlike Onmy-NILTD, these isoforms still encode TM and CYT regions. The Ig domains of all reported teleost NILTs are V-type due to the presence and spacing of two pairs of cysteine residues. The outer cysteines of all NILTs are separated by ~65 aa and are most likely required for the formation of a disulphide bond that stabilizes the tertiary structure of the Ig fold, which usually consists of two β-pleated sheets formed by seven to nine anti-parallel β-strands and connecting loops [149, 153, 221]. Internally, there are two additional cysteines potentially involved in the formation of an additional disulphide bridge. Depending on the NILT Ig domain examined, these internal cysteines are separated by 3, 6, or 7 aa. The influence of these different disulphide bonds and the residues between them may have an impact on the overall shape, charge, and ligand binding properties of the folded protein. Evidence for this proposition has been provided by NILT homology modeling as described in [149]. Using the resolved crystal structure of the NKp44 Ig domain [225], several models of Cyca-NILT1 were generated in order to gain a better prediction of the NILT Ig domain structure. When the NILT structures were compared with NKp44 only minor deviations were observed including an additional strand (D′) in the NILT Ig domain and the presence of a second disulphide bridge formed by the internal cysteines of NKp44, which stabilize the C–C′ β hairpin contributing to the formation of a prominent groove. This second bond was not predicted to exist in Cyca-NILT1 and may be due to differences in

the spacing of the internal cysteines between the two receptors [149]. Further analysis of the predicted Cyca-NILT1 structure revealed that, even in the absence of an internal disulphide bond, a characteristic NKp44 groove-like structure was present and presumed to be stable [149]. Overall, the Cyca-NILT1 groove is relatively non-polar when compared with the charged surface of the NKp44 groove, which could influence the ligand specificity of NILT vs. NKp44 [149, 225]. When the high degree of Cyca-NILT polymorphisms are taken into account and the fact that these polymorphisms are located in and around the groove-like structure it was predicted that Cyca-NILT represents a repertoire of receptors with various specificities and/or ligand affinities [149]. It has also been reported that although NILTs and NITRs are unrelated, their Ig-like domains contain a 21 aa residue consensus motif located in the E and F β-strands [221]. These strands directly face each other on opposite β-sheets in the NKp44 structure and combined with the connecting loop, this may set the boundaries of an accessible pocket required for ligand binding [149, 221].

Teleost NILTs also have characteristic stimulatory and inhibitory signaling potential reminiscent of other vertebrate IgSF immunoregulatory receptors [2, 23, 25-30, 127, 145, 147]. For example, all NILTs described (with the exception of Onmy-NILTD) encode long CYT regions with multiple tyrosine residues. These residues are embedded within the characteristic ITIM and/or ITAMs (Fig. 2.4), which for several immunoregulatory receptor-types are known to engage intracellular phosphatase and kinase pathways, respectively (reviewed in [226]). Cyca-NILT1 is a putative stimulatory receptor since it contains a cytoplasmic ITAM consensus motif, whereas Cyca-NILT2 has two cytoplasmic ITIMs and is likely an inhibitory receptor [149]. Interestingly,

neither NILT-types have a charged residue in their TM segments precluding the possibility they associate with ITAM-containing adaptor molecules such as DAP12 and FcR common γ chain (FcRγ) [227]. Two of the trout NILTs are also predicted to be inhibitory receptors (Onmy-NILT3 and Onmy-NILT4); one is a putative stimulatory receptor (Onmy- NILT2); and the originally described Onmy-NILTD may engage both stimulatory and inhibitory signaling pathways since its CYT region encodes both ITIM- and ITAM-like motifs (Fig. 3) [152, 221]. This unusual arrangement of opposing signaling motifs contained within the same receptor has also been described for zebrafish NITR5 [148], but as with Onmy-NILTD, no information is available regarding how this may manipulate cellular effector responses. It is possible that akin to other receptors encoding dual signaling motifs, teleost receptors such as Onmy-NILTD and NITR5 may play a key role in cellular decision-making as a result of their ability to trigger or inhibit responses due to the selective recruitment of cellular phosphatases and/or kinases [228].

2.4.7.3. NILT expression and function

The expression patterns of both carp and trout NILTs are indicative of their predicted role as regulators of teleost immunity. Various Cyca-NILTs are co-expressed in the spleen, head kidney and trunk kidney, and only Cyca-NILT1 was detected in the thymus with little to no expression of both receptors in the liver, intestine, muscle, and PBL [149]. The expression of Cyca-NILT1 but not Cyca-NILT2 was also observed following extended in vitro cultivation of carp PBLs. Onmy-NILTD expression analysis indicated that the short isoform lacking a TM region (i.e. presumably a secreted protein) has a similar expression pattern as carp NILTs and the long isoform exhibited a more diverse range of tissue expression that included head kidney, spleen, PBL, and gill [221].

Onmy-NILT2, -NILT3, and–NILT4 were also expressed in head kidney, spleen, gill and gut of adults as well as in early developmental stages including eggs and embryos. Expression of these receptors was also evident in the macrophage cell line RTS-11 but not the trout fibroblast line RTG-2 [152].

Based on structural features, signaling potential, expression profiles, and phylogenetic/genomic relationships with mammalian immune receptors, it is likely that teleost NILTs play a decisive role in the regulation of teleost immune cell functions. However, in the absence of any functional data we can only speculate on their putative regulatory roles based on the activities of related receptors such as CMRF-35 leukocyte receptor, NKp44, and TREM [229, 230]. While the establishment of structural and phylogenetic relationships of teleost NILTs with immune receptors in other vertebrates does not automatically imply they share the same functions, this information does provide the basis for designing a logical experimental approach required for the characterization of these unique teleost receptors. Testing the hypothesis that NILTs are the functional orthologues of mammalian CMRF-35/NKp44/TREM will undoubtedly uncover valuable information that can be used to elucidate the role of NILTs in teleost immunity.

2.4.8. Teleost LITRs

2.4.8.1. Discovery of channel catfish LITRs

Ictalurus punctatus LITRs were discovered from an EST library generated from alloantigen-stimulated catfish cytotoxic lymphocytes [2]. The original IpLITRs described (i.e. IpLITR1, IpLITR2, and IpLITR3) are type I TM proteins encoding variable numbers of extracellular C2-like Ig domains, a TM segment, and long or short CYT regions (Fig.

2.6) [2]. Specifically, IpLITR1 encodes a predicted protein consisting of a 14 aa N-terminal secretion signal, a 346 aa extracellular region with four predicted Ig-like domains, a 23 aa TM segment and a long 116 aa CYT region. This CYT region contains two ITIMs, one ITIM-like sequence and an overlapping immunotyrosine- based switch motif (ITSM) centered at Y^{465}, indicating that IpLITR1 most likely initiates inhibitory signaling cascades (Fig. 2.6) [26]. Although variable in their extracellular domain compositions, IpLITR2 (encoding 3 Ig domains) and IpLITR3 (encoding 6 Ig domains) have identical 25 aa TM segments containing a lysine residue and short CYT regions devoid of any signaling motifs (Fig. 2.6) [2]. Consequently, IpLITR2 and IpLITR3 were predicted to be stimulatory receptors requiring association with an adaptor molecule(s) for surface expression and signaling [227]. All three IpLITR prototypes are composed of a membrane distal to proximal ordering of their Ig-like domains, each with a very similar D1 and D2 (i.e. >80% aa identity). However, IpLITR membrane proximal domains are significantly variable between the receptors including IpLITR2 D3, which is only 18–39% identical to other IpLITR domains and IpLITR3 D5 and D6 sharing only 15–25% overall identities with all other domains [227]. A scan of the zebrafish genome database demonstrated that LITRs are not exclusive to catfish, and it is predicted that LITRs are ubiquitous among teleost species [2].

2.4.8.2. Channel catfish LITR sequence and phylogenetic analyses

The IpLITR gene complex is polymorphic, polygenic, and segregation analysis suggested that IpLITR genes are encoded within multiple independently segregating but homologous loci, which was confirmed by mining of the zebrafish genome for IpLITR relatives; i.e. clusters of IpLITR-related exons were identified on multiple zebrafish

chromosomes [2]. It was also hypothesized that IpLITRs are products of multiple gene duplication and translocation events from an ancestral gene, but it is not known if the different IpLITR loci represent functionally redundant or distinct gene clusters. However, as described below, IpLITRs are distantly related to a variety of functionally and genomically distinct mammalian IgSF members (i.e. FcRs and LRC-encoded proteins), suggesting that during vertebrate evolution certain paralogous loci could have evolved to give rise to an array of IgSF immunoregulatory receptors with variable functions.

Database searches identified multiple immunoregulatory receptor genes from several vertebrate species (i.e. mammals, birds, and amphibians) as potential IpLITR relatives making it difficult to pinpoint true homologues for this family. These matches included several members of the LRC as well as FcRs and FCRLs [2]. Notably, IpLITRs were not closely related to any of the previously described teleost IgSF immunoregulatory receptors described including NITRs, NILTs,and IpFcRI (Table 2.1).

Support for the relationship of IpLITR Ig-like domains with those domains from a broad range of vertebrate IgSF members was also deduced from sequence alignments and phylogenetic analyses [2]. The membrane distal IpLITR D1 and D2 Ig- like domains preferentially clustered with those encoded within FcR and FCRL proteins, whereas IpLITR membrane proximal Ig-like domains exhibited a closer relationship with IgSF members encoded within the LRC [2]. While sequence comparisons alone do not unequivocally establish IpLITRs as distant relatives of any particular IgSF members, the arrangement of Ig-like domains within IpLITRs provides some support for the hypothesis that certain mammalian IgSF members may have evolved from a common ancestor that has since undergone multiple rounds of duplication and homologous recombination

events [2, 149, 231]. Nonetheless, further studies are required to prove whether or not IpLITRs share true orthologous relationships with other vertebrate IgSF members.

2.4.8.3. Channel catfish LITR gene expression

IpLITR messages are highly expressed in the hematopoietic pronephros and mesonephros with lower levels in the spleen, gill, and heart as determined from Northern blotting [2]. Similar results were observed using both IpLITR1 and IpLITR2-specific probes and in both cases multiple hybridizing bands were detected. RT-PCR indicated that IpLITR1 (putative inhibitory) and IpLITR2 (putative stimulatory) are coordinately expressed in a variety of different catfish cells and tissues (e.g. PBL, spleen, kidney, gill, heart, muscle, liver, intestine, thymus, macrophages, B cells, T cells, and NK-like cells) but no expression was detected in the fibroblast CCO cell line [2]. IpLITR expression analysis also led to the cloning of several additional IpLITR1-like and IpLITR2-like transcripts, which were presumably amplified due to cross-hybridization of the primers with highly related IpLITR genes and/or alternatively spliced products [2]. These variant IpLITR sequences were co-expressed by myeloid and lymphoid catfish cell lines and they varied in the number of Ig-like domains encoded, and the length of their CYT regions. Expression of the inhibitory and stimulatory IpLITR prototypes was also examined in activated catfish PBL and cytotoxic T lymphocytes up to 12 days post-stimulation with mitogens and/or allogeneic targets [3]. Neither bacterial lipopolysaccharide nor concanavalin A induced the expression of IpLITRs in catfish PBLs, but when stimulated with alloantigen, co-expression of both IpLITR-types was observed on day 8 and day 12 and then again on days 2, 4, 6, and 8 after re-stimulation with the same alloantigen [3]. Similar expression results were also observed with clonal cytotoxic lymphocyte cell lines

[3]. As mentioned earlier, RT-PCR primers designed for the IpLITR1 and IpLITR2 prototype sequences captured the expression of multiple variations of these prototype sequences. In fact, a large repertoire of inhibitory and stimulatory IpLITR-types were identified when these expressed transcripts were cloned and sequenced from alloantigen stimulated lymphocytes, increasing the range of sequenced IpLITR cDNAs from three original genes to forty [3]. These novel IpLITRs had variable numbers of extracellular domains (i.e. from 2 to 7 Ig-like domains) and the majority of these sequences were IpLITR2-like (i.e. putative stimulatory receptors with a charged TM segment and short CYT regions).

2.4.8.4. Channel catfish LITR structure and predicted binding partners

IpLITRs exhibit a membrane distal to proximal ordering of their C2 Ig-like domains and although some IpLITRs encode more domains than others, they all share similar membrane distal regions encoding the D1 and D2 [2, 3]. Each Ig-like domain is ~75 aa long and contains two conserved cysteine residues for stabilization of the tertiary structure of the Ig-fold that in the case of IpLITRs consists of seven to eight anti-parallel β-strands and connecting loops. Interestingly, although the D1D2 configuration is conserved among IpLITRs, these domains also represent the most variable regions among all IpLITRs examined [3]. Multiple aa substitutions are present in the membrane distal D1 and D2 regions, which are clustered at specific locations within these domains. Comparatively, fewer regions of aa variability were detected in the membrane proximal domains (i.e. D3 and D4). This led to the hypothesis that like other known IgSF members that use their D1D2 for ligand binding (e.g. FcRs, KIRs, and LILRs), the D1D2 region of these fish receptors may serve as the ligand-binding subunit, prompting the search for

conserved binding sites within this region. Specifically, using the D1D2 sequences of IgSF members that have been co-crystallized with their respective ligands, sequence comparisons were performed in an attempt to search for any putative/conserved IpLITR ligand-binding site(s). Using multiple sequence alignments and comparative homology modeling, key residues involved in the LILRB1-MHC I interaction in humans [10, 232] were mapped at similar positions within the D1D2 of representative IpLITRs [3]. Surprisingly, six of the residues located in the first Ig domain of LILRB1 known to make contact with the MHC-I α3 domain were either identical or conserved among several IpLITR D1 sequences [3, 10]. The location of these residues was between the predicted C and C′ β-strands found at the membrane distal tip of IpLITR D1 with an additional residue preceding the second conserved cysteine residue within this domain. Importantly, these positions are identical to the known MHC I α3 contact residues within LILRB1 [10]. Since human LILRB1 D1D2 also interacts with β-2-microglobulin (β2M) [10, 232], the aa sequence was investigated to determine if β2M-contact sites were present within the IpLITR D1D2. While this interaction interface was not as recognizable as the heavy chain contact site (i.e. MHC-I α3), several residues were identified as potential β2M contacts [3]. This co-evolution of IpLITRs with their teleost MHC I targets may play some role in the variability observed within this predicted binding site if indeed catfish MHC I is the target ligand for these receptors.

Although the identification of a putative MHC-I-binding site in the IpLITR D1D2 is interesting and warrants further investigation, this is only based on sequence alignments and comparative modeling. In the absence of any supporting data this simply remains a hypothesis, and the search for IpLITR ligands must not be restricted to MHC-I

molecules. Discovering the ligands for any newly identified receptor represents a formidable challenge, particularly when the receptor is a member of a highly diversified polymorphic and polygenic gene family. Predictions based on sequence and structural analyses alone can also be misleading as was demonstrated recently from studies of the CHIRs. While encoded within the chicken LRC and historically predicted to be functionally similar to mammalian NK cell receptors, many of these proteins have now been characterized as high affinity Fc receptors for IgY [233]. Another example comes from a member of the FCRL family (FCRL6): despite having 'Fc-related' in its name, this receptor directly binds HLA-DR (an MHC-II molecule) [234]. Recently, it has also been demonstrated by the same group that the B-cell expressed FCRL5 binds to a viral encoded MHC-I- like molecule referred to as immunoevasin [235]. Combined with the current information that the ligands for mammalian IgSF immunoregulatory receptors are extremely diverse (e.g. sialic acids, collagen, albumin, bacterial and viral pathogen products, MHC-I molecules, Fc regions of Ig, and others; reviewed in [25, 49], a range of experimental approaches are necessary for identifying any potential ligands that may be targeted by polymorphic and polygenic teleost IgSF immunoregulatory receptors such as IpLITRs.

2.4.8.5. Characterization of stimulatory IpLITR-types

Current research in our lab has focused on identifying the intracellular signaling pathways that are recruited by the various IpLITR- types [156, 157]. Putative stimulatory IpLITRs contain charged TM segments due to the presence of a single lysine residue within this region [2] and it was predicted that these IpLITR-types recruit ITAM-containing signaling adaptors [156]. To address this hypothesis experimentally, HEK

293T cells were transiently co-transfected with haemagglutinin (HA) epitope-tagged teleost ITAM-containing adaptor molecules in conjunction with putative stimulatory IpLITR-types [156]. Then, using co-immunoprecipitation and flow cytometric analysis it was demonstrated that putative stimulatory IpLITRs preferentially associated with catfish FcRγ-like adaptor proteins (e.g. IpFcRγ and IpFcRγ-L), which significantly increased cell surface expression of the receptor [156]. Enhanced surface expression in the presence of these teleost FcRγ-like adaptor proteins indicated that the assembly of stimulatory IpLITRs with an appropriate adaptor is required for the expression of a functional signaling sub-unit [156]. Neutralization of the negatively charged aspartic acid residue within the FcRγ-like TM segment completely abrogated the enhanced IpLITR surface expression, which correlated with failure of the receptor to associate with this adaptor [156]. Surprisingly, when the TM segment of the stimulatory IpLITR was neutralized by mutating the lysine into an alanine, association with the FcRγ-like adaptor protein still occurred, which correlated with maintained surface expression of the receptor–adaptor complex [156]. This is contrary to what has been reported for stimulatory receptor–adaptor associations in other vertebrates [132, 145, 154, 155, 227] and raises the possibility that the presence of the lysine residue within the TM region of IpLITRs is not a pre-requisite for their signaling potential. Other residues within the IpLITR TM segment may influence the affinity and specificity of these interactions, and further mutagenesis experiments are required to define the precise molecular interactions that influence IpLITR-mediated stimulatory signaling capabilities. With a basic understanding of IpLITR adaptor recruitment in place, ongoing studies focused on determining the intracellular signaling cascades that are initiated following ligation of the IpLITR and the

influence that this has on cellular responses. An epitope-tagged chimeric receptor construct was created by fusing the EC region of IpLITR2.6b to the TM region and CYT tail of IpFcRγ-L. This chimera was stably expressed in a rat basophilic leukemia (RBL)-2H3 cell line and, following cross-linking of the surface receptor with an anti-hemagglutinin (HA) monoclonal antibody (mAb) or opsonized microspheres, the chimeric teleost receptor induced cellular degranulation and phagocytic responses, respectively. Site-directed mutagenesis of the immunoreceptor tyrosine-based activation motif encoded within the CYT confirmed that these functional responses were dependent on the phosphorylated tyrosines within the ITAM. Using a combination of phospho-specific antibodies and pharmacological inhibitors, it was also demonstrated that the IpLITR/IpFcRγ-L-induced degranulation response requires the activity of PI3K, PK-C, and MAPKs but appears independent of the c-Jun N-terminal kinase and p38 MAP kinase pathways [157].

2.5. Concluding Remarks

Families of IgSF immunoregulatory receptors that bind similar ligands are commonly encoded as clusters of genes on various chromosomes. For example, receptors that interact with the Fc portion of antibodies are encoded on human Chr 1q21–23, whereas receptors involved in the surveillance of MHC I antigens, such as KIRs and LILRs, are clustered in a region on Chr 19q13.4 referred to as the LRC. Studies in other vertebrates such as mice, birds, and amphibians have revealed that genomic regions containing a high density of IgSF members reminiscent of the human LRC and FcR complexes are conserved among distinct vertebrate species. As increasing numbers of IgSF members are being described, hypotheses into the common evolutionary origins of

these immunoregulatory receptors continue to emerge. While the discovery of multiple immunoregulatory receptors belonging to the IgSF across vertebrate taxa provides researchers with an opportunity to reconstruct the phylogeny of vertebrate receptor networks, phylogenetic relationships alone are not sufficient for truly appreciating the roles that these receptors play in the regulation of immunity. In particular, many of the IgSF members recently described in teleost (i.e. NITRs, FcRs, DICPs, MDIRs, NILTs, and LITRs) remain to be functionally characterized and defining their relatedness to other vertebrate IgSF members is difficult. To date, teleost NITRs are the best studied of these multigene families, which have provided novel information into their signaling capabilities, structural features, and binding/recognition characteristics; reviewed in [183].

I anticipate that in the near future, a clearer picture will emerge as to how the various teleost IgSF members work to control and coordinate a range of immune cell effector functions in fish. Since members of these receptor families are often co-expressed on the same immune cell subpopulations, studying the interplay between teleost IgSF superfamily networks presents a daunting but exciting opportunity for developing an understanding of the intricacies of complex immunoregulatory receptor networks. Progress in the understanding of teleost immune receptors will provide valuable insights into the workings of not only teleost immune responses but also those of other vertebrates including mammals.

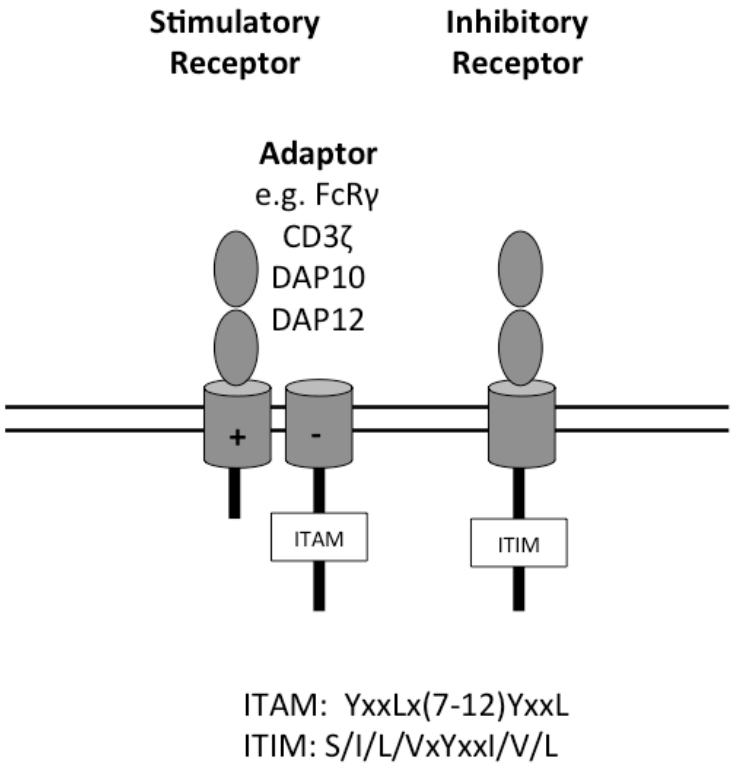

Figure 2.1. A schematic repesentation of stimulatory and inhibitory immunoregulatory receptor types. The stimulatory receptor is contains an extracellular region, a transmembrane region containing a charged aa residue, and a short cytoplasmic region that lacks motifs capable of signaling. In order to initiate cellular signaling, the stimulatory receptor associates with a membrane bound adaptor molecule through an interaction with a negatively-charged aa in the transmembrane region. The adaptor molecule iniates cellular signaling via a cytoplasmic ITAM motif. The inhibitory receptor is characterized by it extracellular region, uncharged transmembrane region, and long cytoplasmic region, which is capable of initiating inhibitory signals via one or more ITIMs. Below the schematic are the canonical aa motifs that characterize the ITAM and ITIM.

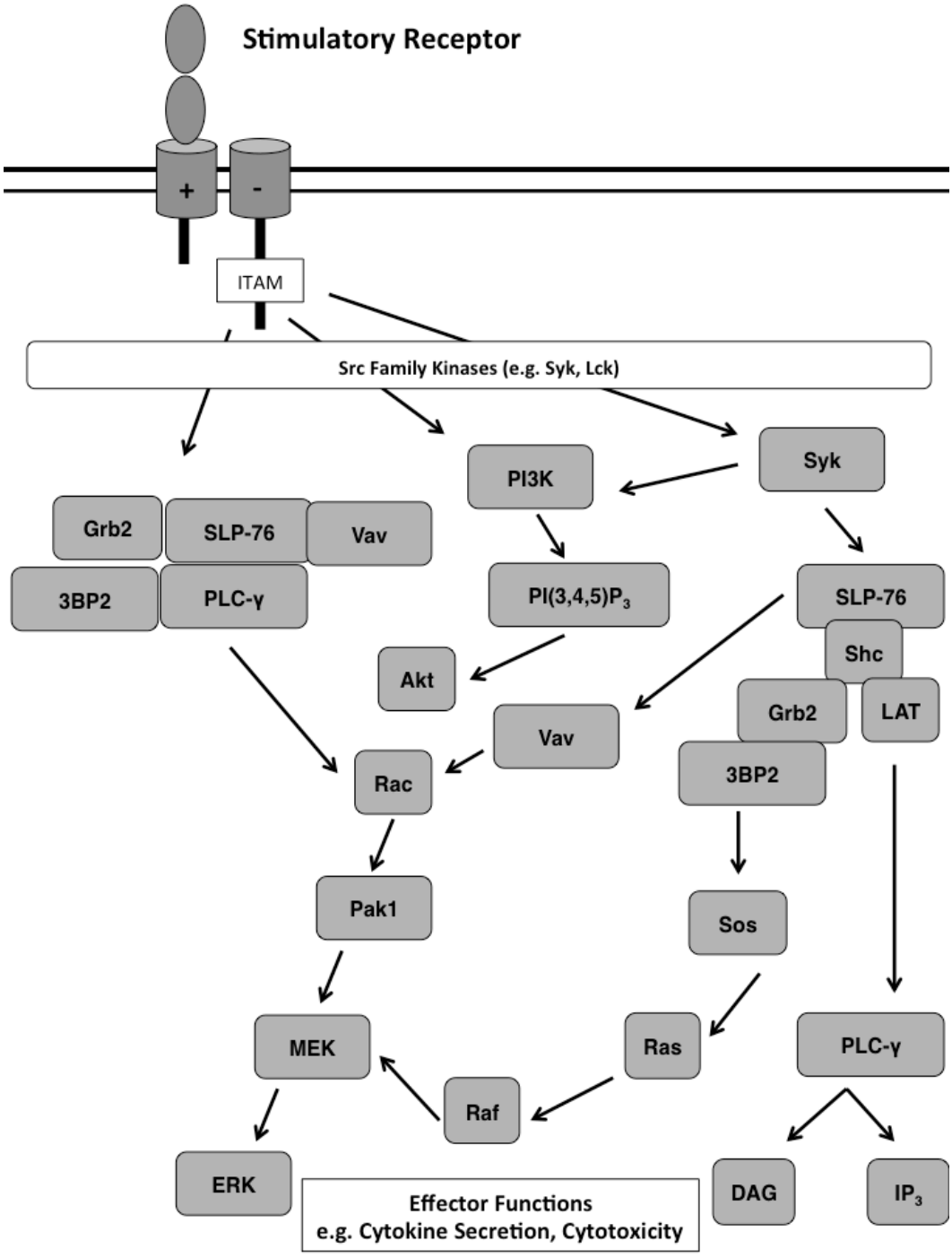

Figure 2.2. A **schematic representation of a leukocyte effector signaling pathway.** Upon engagement, the stimulatory receptor signals through an ITAM-bearing adaptor molecule. The tyrosine residues in the ITAM are phosphorylated by SFKs and this leads to the initiation of a kinase cascade that utilizes a complex network of molecules. Selected signaling molecules are presented here but additional molecules are involved in a comprehensive effector response. This figure is adapted from [44]

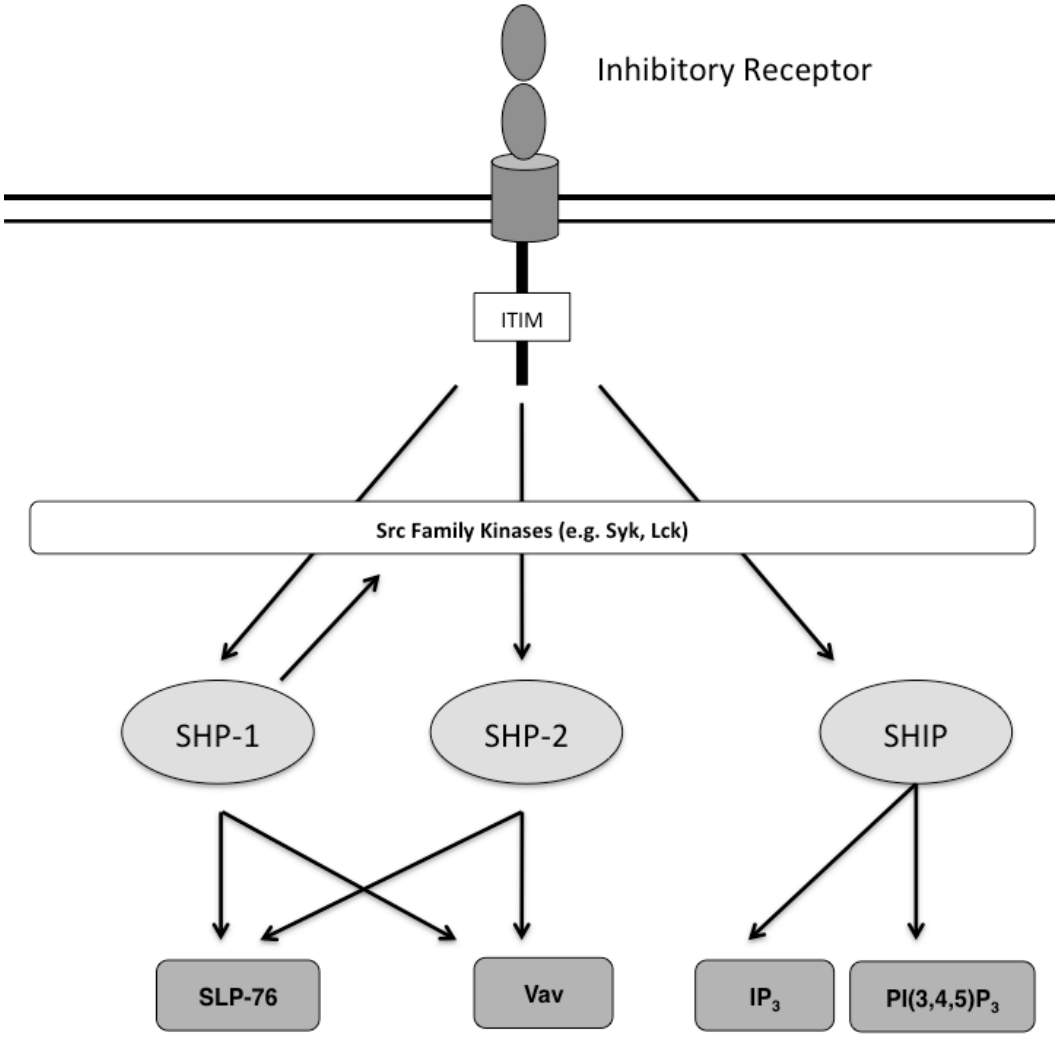

Figure 2.3. A **schematic representation of inhibitory receptor signaling.** The ITIM of the inhibitory receptor is phosphorylated by SFKs following engagement with its respective ligand. The phospho-ITIM then recruits SHP-1, SHP-2, and/or SHIP. These phosphatases then dephosphorylate their respective substrates (e.g. SLP-76, Vav, IP_3, $PI(3,4,5)P_3$, as well as SFKs. This enzymatic activity leads to a shut down of the leukocyte effector response.

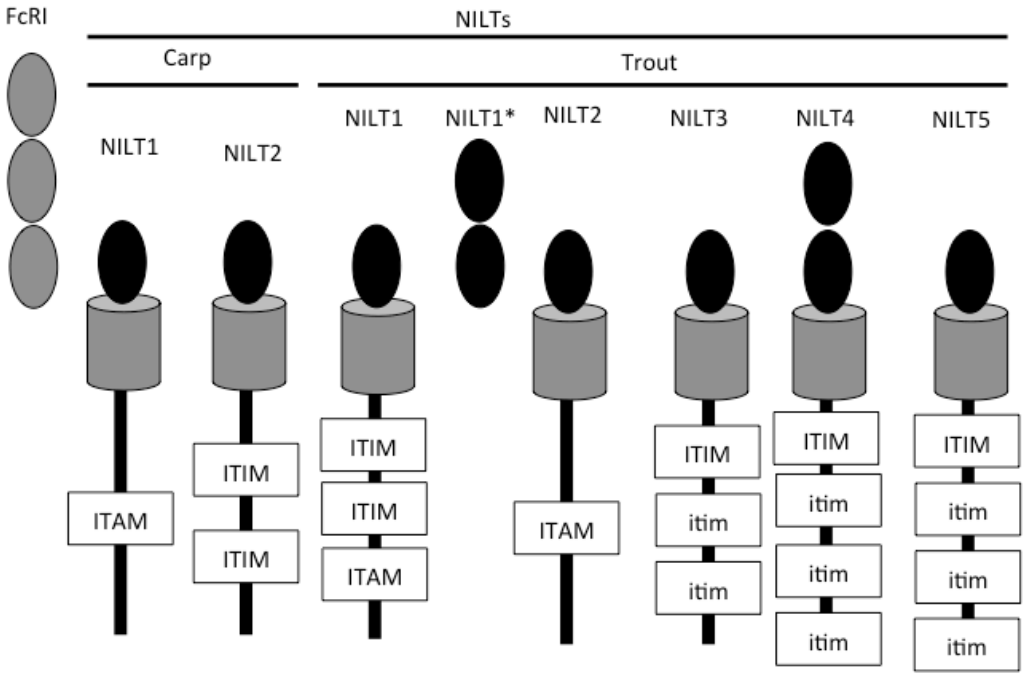

Figure 2.4. Schematic representation of the structural characteristics of FcRl and NILTs. The extracellular Ig domains are either C2-type (grey) or V-type (black). The TM region is indicated by the cylinder and the cytoplasmic tail may contain an ITAM, ITIMs, or ITIM-like motifs ('itim').

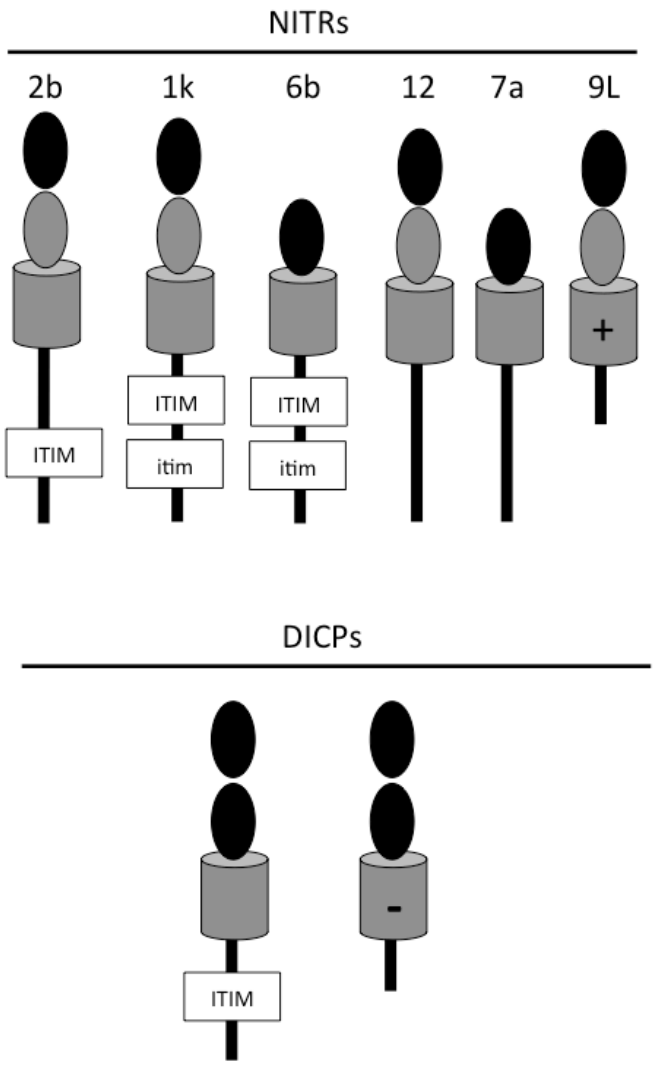

Figure 2.5. Schematic representation of bony fish NITRs and DICPs. Selected NITRs and DICPs from zebrafish are presented. Extracellular Ig domains are indicated as being either V-type (black) or C2-type (grey). A TM region is indicated with the cylinder, which may encode an aa with a positive (+) or negative charge (-) as indicated. Long cytoplasmic tails may encode ITIM motifs ,as indicated by 'ITIM', or ITIM-like motifs, as indicated by 'itim'.

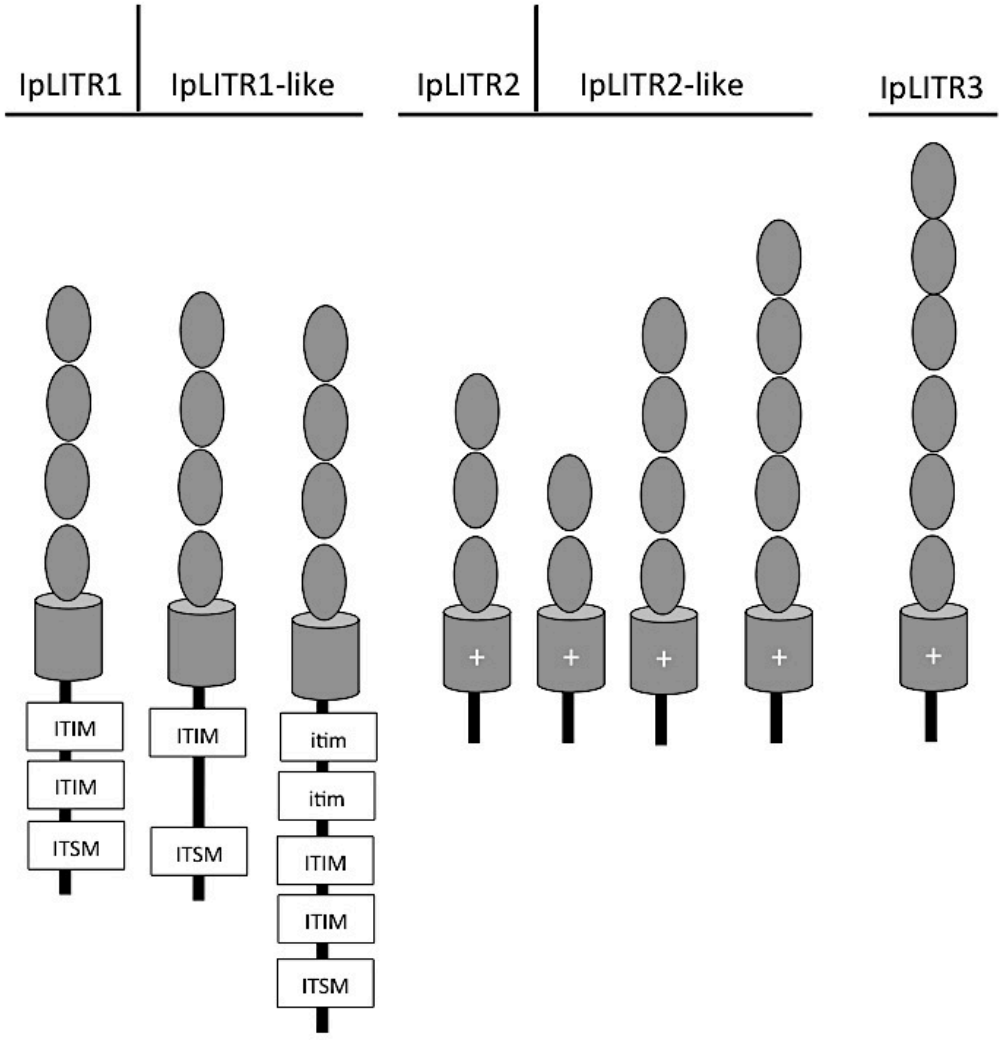

Figure 2.6. A Schematic representation of the channel catfish LITRs. LITRs contain variable numbers of C2-type Ig domains as indicated by the grey ovals. The TM region is indicated by the cylinder and may encode for a charged amino acid residue, as indicated by the (+). Longer cytoplasmic tails contain signaling motifs including ITIMs, ITIM-like motifs ('itim'), or ITSMs.

Table 2.1. Database searches for teleost IpLITR-like sequences

Teleost Species	Common Name	Top Hits	Accession	E value	% Identity	% Positives	Coverage
Ictalurus punctatus	Channel catfish	**IpLITR1**	AAW82352	0	100	100	368/368 (100%)
Danio rerio	Zebrafish	novel protein	XP_694305	5e -47	38	55	313/368 (85%)
Danio rerio	Zebrafish	sialoadhesin-like	XP_692355	2e -52	39	56	325/368 (88%)
Danio rerio	Zebrafish	high affinity FcRIa, CD64-like	XP_001345071	8e -41	35	51	315/368 (86%)
Danio rerio	Zebrafish	**predicted IpLITR TS32.15 L1.1a-like**	XP_002666999	1e -16	37	55	165/368 (45%)
Danio rerio	Zebrafish	myelin-associated glycoprotein-like	XP_001920377	8e -16	28	45	285/368 (77%)
Danio rerio	Zebrafish	predicted FCRL 2-like	XP_002660646	2e -13	62	88	179/368 (49%)
Danio rerio	Zebrafish	CEACAM1-like	XP_001333522	4e -13	32	48	170/368 (46%)
Danio rerio	Zebrafish	pregnancy specific glycoprotein-like	XP_002667463	1e -12	30	46	248/368 (67%)
Salmo salar	Atlantic salmon	high affinity FcRI precursor	ACN10664	3e -11	26	45	237/368 (64%)
Tetradon nigroviridis	Green pufferfish	unamed protein	CAF97032	3e -10	28	43	255/368 (69%)
Paralichthys olivaceus	Halibut	IgD	BAB41204	5e -07	23	41	342/368 (93%)
Salmo salar	Atlantic salmon	platelet endothelial cell adhesion molecule	NP_001133571	9e -07	30	44	175/368 (48%)
Takifugu rubripes	Tiger pufferfish	Neural cell adhesion molecule L1	Q98902	4e -04	21	36	291/368 (79%)
Salmo salar	Atlantic salmon	Neural cell adhesion molecule	NP_001134287	7e -04	24	40	199/368 (54%)
No matches for:	*Cyprinus carpio* (carp), *Epinephelus coioides* (grouper), *Fundulus heteroclitus* (killifish), *Gasterosteus aculeatus* (stickleback), *Oncorhynchus mykiss* (trout), *Oreochromis niloticus* (tilapia), *Oryzia latipes* (medaka), *Sparus aurata* (sea bream)						
Ictalurus punctatus	Channel catfish	**IpLITR2**	AY885644	0	100	100	257/257 (100%)
Danio rerio	Zebrafish	sialoadhesin-like	XP_692355	3e -40	43	58	244/257 (95%)
Danio rerio	Zebrafish	predicted: novel protein	XP_694305	1e -33	39	55	243/257 (94%)
Salmo salar	Atlantic salmon	high affinity FcRI precursor	ACN10126	3e -06	27	45	102/257 (40%)
Danio rerio	Zebrafish	high affinity FcRIa, CD64-like	XP_001345071	2e -29	38	51	235/257 (91%)
Tetradon nigroviridis	Green pufferfish	unamed protein	CAF97032	2e -15	30	44	255/257 (99%)
Danio rerio	Zebrafish	**predicted IpLITR TS32.15 L1.1a-like**	XP_002666999	1e -12	36	58	126/257 (49%)
Danio rerio	Zebrafish	myelin-associated glycoprotein-like	XP_001919202	9e -06	27	43	241/257 (94%)
Danio rerio	Zebrafish	junctional adhesion molecule 2	NP_001121766	2e -04	32	41	106/257 (41%)
Danio rerio	Zebrafish	CEACAM1-like	NP_001107266	8e -04	23	40	228/257 (89%)
No matches for:	*Cyprinus carpio* (carp), *Epinephelus coioides* (grouper), *Fundulus heteroclitus* (killifish), *Gasterosteus aculeatus* (stickleback), *Oncorhynchus mykiss* (trout), *Oreochromis niloticus* (tilapia), *Oryzia latipes* (medaka), *Paralichthys olivaceus* (halibut), *Sparus aurata* (sea bream), *Takifugu rubripes* (tiger pufferfish)						

Notes:
(a) Searches of the non-redundant protein sequence databases were performed using blastp at http://blast.ncbi.nih.nin.gov/Blast.cgi on Jan 5, 2011.
(b) The 368 amino acid sequences encoding the four Ig-like domains of channel catfish LITR1 (AAW82352) and the 257 amino acid sequence encoding the three Ig-like domains of channel catfish LITR2 (AY885644) were used as a queries to search each of the teleost databases.
(c) For each teleost species the top scoring match is listed and additional hits are listed if they represent a different match than the top hit.
(d) Matches are listed from lowest to highest E values and only E values <10e−04 are reported.
(e) Coverage refers to the number of amino acids that match the catfish LITR1 (AAW82352) and LITR2 (AV885644) sequence queries (including gaps).
(f) Coverage values reported in bold-type indicate exclusion of gaps from the analysis.

CHAPTER III

MATERIALS AND METHODS

3.1. Cells and Antibodies.

3.1.1. Cells

HeLa, human embryonic kidney (HEK) 293T, Thymidine kinase negative (TK-), and Phoenix cells were grown at 37 ^{o}C and 5% CO_2 in DMEM/High Glucose (HyClone) supplemented with 2mM L-glutamine (Invitrogen Life Sciences), 100 Units/mL penicillin (Invitrogen Life Sciences), 100 mg/mL streptomycin (Invitrogen Life Sciences), 1 mM sodium pyruvate (Invitrogen Life Sciences), 1% MEM non-essential amino acid solution (Invitrogen Life Sciences), and 10% heat-inactivated fetal bovine serum (characterized; Hyclone).

YTS cells, a human NK-like lymphocytic cell line, were cultured in Iscove's media containing 15% FBS, 50 μM β2-mercaptoethanol (ME), 2 mM L-glutamine and supplemented with 1 μg/ml puromycin (Invitrogen Life Sciences) after transduction. These cells lack surface expression of KIR and LILR receptors and are stimulated to induce cytotoxicity through the engagement of CD28 to B7 on a target cell [236]. Prior to use, culture media was filter sterilized using 0.22mm filter units (Corning).

NK92 cells were cultured in Iscove's media with 10% fetal bovine serum (FBS; Hyclone), 50 μM β2-ME, and 2mM L-glutamine (Invitrogen Life Sciences) supplemented with 100 U/ml human recombinant IL-2 (TECIN^{TM}, Biological Resources Branch, DCTC, NCI-Frederick Cancer Research and Development Center). The NK92 cell line was obtained from Dr. Deborah Burshtyn and purchased from American Tissue

Culture Collection (ATCC; CRL-2407). NK92 cells are a human NK-like lymphocytic cell line that lack expression of the KIR family of receptors but do express natural killer receptors (NKRs) [237].

The MHC-I-deficient B-cell lymphoma target cell line 721.221 was maintained in Iscove's media with 10% FBS and 2 mM L-glutamine. 721.221 cells expressing HLA-Cw3 or HLA-Cw15 were maintained in 721.221 media supplemented with 0.5 μg/mL geneticin (Invitrogen Life Sciences).

Mouse NK cells were derived from the splenocytes of C57BL/6 mice as previously described [238]. Briefly, spleens were isolated from male C57BL/6 mice and mashed through a cell strainer with 40μm nylon mesh (BD Biosciences). Isolated cells were washed with 50 mL of Dulbecco's-PBS (D-PBS) twice and resuspended in RPMI media (Invitrogen, Life Sciences) with 10% FBS, 50 μM β2-ME, 2 mM L-glutamine, 100 Units/mL penicillin, 100 mg/mL streptomycin, and 1000 U/mL human recombinant interleukin 2 (IL-2) (BD Biosciences). Washed cells were passaged through a nylon wool column (30 mg of nylon wool (sterile; Polysciences Inc.) in a a 30 mL syringe (BD Biosciences) and then collected in 1mL media/1x10^6 cells. On day 3 the plastic adherent cells were retained and restimulated with rIL-2. Purity was assessed by flow cytometry on day 6 with anti-NK1.1, a mAb that detects the NK cell-associated marker CD161, and phycoerythrin-coupled anti-mouse CD3, which detects T cells (Pharmingen, San Diego, CA). Cells were >99% $NK1.1^+$ and ranged from 10-30% $CD3^+$. Cytotoxicity assays were performed on day 7 or 8. All work associated with mouse spleen collection was approved by the Health Research Ethics Board at the University of Alberta.

3B11 is a cloned autonomous B cell line generated from an outbred catfish by mitogen stimulation [239]. Catfish cells lines were grown at 27°C in AL-3 medium consisting of equal parts AIM-V and L-15 (both from Life Technologies) adjusted to catfish tonicity with 10% (v/v) deionized water and supplemented with 1 μg/ml $NaHCO_3$, 50 U/ml penicillin, 50 μg/ml streptomycin, 20 μg/ml gentamicin (Invitrogen Life Sciences), 50 μM β2-ME, and 3% heat-inactivated, pooled, normal catfish serum [166].

3.1.2. Antibodies

The following antibodies were used in this thesis: anti-HA mAb clone HA.C5 (Abcam), goat anti-mouse IgG (H + L)-FITC (Cedarlane Laboratories Ltd.), HRP-conjugated goat anti-HA polyclonal antibody (GenScript Corp.), anti-phosphotyrosine mAb 2C8 (Santa Cruz Biotechnology, Inc.), biotin-conjugated anti-phosphotyrosine mAb 4G10 (Upstate Biotechnology), anti-FLAG mAb M2 (Stratagene), anti-FLAG M2 mAb peroxidase conjugated (Sigma–Aldrich), goat anti-mouse IgG (H + L)-PE (Beckman Coulter), anti-KIR2D mAb (LIG-1) was provided by Dr. Deborah Burshtyn ([240]. DX27 (IgG2a) is an anti-KIR2DL2/L3/S2 antibody and was provided by Dr. Lewis Lanier (UCSF, San Franciso, CA; [241]. (Both LIG-1 and DX27 were generous gifts from Dr. Deborah Burshtyn). W6/32 (IgG2a) a pan-HLA-reactive monoclonal antibody (mAb), L243 (IgG2a) an anti-HLA-DR mAb and the isotype control IgG2a (51.1) were all purified by protein G-Agarose from hybridomas obtained from ATCC. Anti-hemagglutinin (HA) mAb and phycoerythrin (PE)-conjugated goat anti-mouse IgG were purchased from Cedarlane Laboratories. The anti-Csk rabbit antiserum was kindly provided by Dr. André Veillette (Institut de recherches cliniques de Montreal, Montreal,

Canada). Horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG was purchased from Bio-Rad.

Sodium pervanadate, a general competitive inhibitor for protein tyrosyl phosphatases was solubilized in water (NEB Inc.). The SHP-1/2 inhibitors, sodium stibogluconate and PTP I inhibitor, were solubilized in DMSO and used at the concentrations indicated (EMD Millipore).

3.2. Cloning and Expression Constructs

3.2.1. Generation of 'native' epitope-tagged IpLITR

Several putative inhibitory IpLITRs have been cloned from alloantigen-stimulated catfish cytotoxic T lymphocytes [3]. These cDNAs were cloned into the pCR4-TOPO vector (Invitrogen) and provided as a kind gift from Dr. Norman Miller (University of Mississippi Medical Center) as templates for generating an epitope-tagged receptor. Ten nanograms of the pCR4-TOPO vector containing the sequence for TS32.17 L1.1b (NCBI accession number: ABI16050) was amplified with pDISPLAY L1.1b/*Sma*I forward (Fwd) and pDISPLAY L1.1b/*Sal*I reverse (Rvs) primers (Table 3.1) using 0.4 U of Phusion High-Fidelity DNA polymerase (Finnzymes) in 20 μL reactions according to manufacturer's recommended instructions. Cycling parameters were as follows: 40 seconds (s) at 98^{o}C, 30 cycles of 98^{o}C for 20s, 64^{o}C for 25s, 72^{o}C for 30s, and a final extension step for 7min at 72^{o}C. Note: The generation of all expression constructs in this thesis was performed using Phusion High-Fidelity polymerase. Reactions were then separated on a 1.0% TAE-agarose gel, and visualized by staining with ethidium bromide solution (50 μg/l), and the PCR product (~1500bp) was excised, gel purified (Qiagen) and cloned into pJET1.2/blunt using the blunt-end protocol (Fermentas). Cloning reactions

were then transformed into NEB 10-beta *E. coli* (New England BioLabs) and positive clones identified by performing colony PCR with the pJET1.2 Fwd and Rvs sequencing primers (Table 3.1). A randomly selected positive colony was grown overnight in 8 ml of LB-ampicillin (Bio-Rad) (100 μg/ml) and the plasmid was isolated using the Qiagen miniprep kit according to the manufacturer's instructions. Two micrograms of the purified plasmid was double digested for 2 h with 10 U of *Sma*I/ *Sal*I and the insert isolated and then ligated into the pDISPLAY vector (Invitrogen Life Sciences) using T4 DNA ligase (Fermentas) and a 2.5 h incubation at 25^{o}C followed by an overnight incubation alternating between 4^{o}C and 16^{o}C every 45 min. Positive pDISPLAY-IpLITR TS32.17 L1.1b clones were identified by restriction digestion and purified plasmids were sequenced to verify the cDNA sequence and to ensure that the construct was in-frame without any base pair changes. 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.

3.2.2. KIR/IpLITR Chimeric constructs

To characterize the signaling potential of putative inhibitory catfish LITRs I constructed 'chimeric' expression constructs consisting of the EC and TM segment of human KIR2DL3 fused to the tyrosine-containing CYT region of two different IpLITR receptors. Briefly, the full-length cDNA for KIR2DL3 (NCBI accession number: U24074), previously ligated in the pSC65 vector [93], was subcloned into pSPORT-1 (Invitrogen Life Sciences) using *Sal*I and *Not*I digests. pSPORT-1/KIR2DL3 was then amplified with forward and reverse primers designed to amplify around the vector in order to introduce a *Kpn*I restriction site just after the TM segment of KIR2DL3 (i.e. bp

position 795 of the open reading frame). This strategy allowed for the direct fusion of the CYT regions of IpLITR cDNAs directly after the EC region and TM segment of KIR2DL3. Catfish LITRs used for the CYT region amplifications (NCBI accession numbers: ABI16050 and ABI16051) were previously cloned into pCR4-TOPO (Invitrogen Life Sciences). The IpLITR CYT regions were amplified using specific forward primers with a 5' phosphate (to facilitate fusion immediately after the 795 bp of KIR2DL3) and a reverse primer that introduced a *Kpn*I restriction site immediately following the native IpLITR CYT 'stop codon; TAG'. Products were gel purified, digested with *Kpn*I and then ligated into the pSPORT-1/KIR2DL3 to create a chimeric cDNA construct [KIR/IpLITR]. Three different constructs were generated: KIR/IpLITR1.2a contains the entire CYT region of ABI16051 (i.e. 1170–1326 bp; amino acid 391–441); KIR/IpLITR1.1b contains the entire CYT region of ABI16050 (i.e. 1167–1533 bp; amino acid 389–511); and KIR/IpLITR1.1b TR contains a truncated region of the CYT of NCBI accession number: ABI16050 (i.e. 1167–1398 bp; amino acid 389–466). During the sequencing KIR/IpLITR1.1b, a frame-shift mutation was identified in one of the clones that introduced a premature stop codon resulting in a mutated receptor that was devoid of any tyrosine residues in its short 28 amino acid CYT. Consequently, this construct was chosen as a negative control termed KIR/IpLITR ΔCYT. All chimeric constructs were then amplified with PCR primers to facilitate *Sal*I/*Not*I or *BamH*I/*Not*I restriction cloning into the final destination vectors pBABE (Cell Biolabs, Inc) or pMX-puro (Ariad Pharmaceutical), respectively. These plasmids are amphotropic (i.e. able to infect and be expressed in multiple species and cell lines) retroviral expression plasmids that facilitate stable expression of proteins in transformed NK-like cell lines such as

human YTS cells and transient expression in HeLa and HEK 293T. For the generation of recombinant vaccinia virus, each KIR/IpLITR construct was subcloned into the pSC66 vector using *Sal*I/*Not*I restriction sites. The pSC66 vector is a modification of the pSC65 vector, which includes additional cut sites. The pSC66 vector was used to express the chimeric constructs under the control of the early/late synthetic poxviral promoter [242]. Prior to cellular transfections, all KIR/IpLITR constructs were sequenced to verify the cDNA sequence and to ensure that the constructs were in-frame without any base pair changes.

3.2.3. Site-Directed Mutagenesis of KIR/IpLITR1.1b TR.

Tyrosine-to-phenylalanine mutants within the CYT region of KIR/IpLITR1.1b TR were generated by site-directed mutagenesis using the QuickChange Lightning site-directed mutagenesis kit (Stratagene). Primers used to generate KIR/IpLITR 1.1b TR FY were:

5’-CTTCAGACCGATGAGCACATTTTTGACACTGTGGA-3’ and its reverse complement. Primers used to generate KIR-LITR 1.1b TR YF were:

5’-GAACTCAGTGGGGCCGTTTT TGCACAGGTCAT-3’ and its reverse complement. Primers used to generate KIR-LITR 1.1b TR FF were 5’-CTTCAGACCGATGAGCA- CATTTTTGACACTGTGGA-3’ and its reverse complement followed by

5’-GAACTCAGTGGGGCCGTTTTTGCACAGGTCAT-3’ and its reverse complement.

3.3. Development of Recombinant Vaccinia Viruses

All pSC66-KIR/LITR constructs and an empty pSC66 vector were recombined with the vaccinia virus strain Western Reserve (WR) as described [243]. Vaccinia viruses encoding KIR2DL3 and dominant-negative (DN)-SHP-1 have been described, previously named cl6, and HCPC453S, respectively [50]. All rVV were propagated in the TK- cells, released from the cells by sonication, and enriched by spinning through a 36% sucrose cushion [244]. Titers in plaque-forming units were determined in TK-cells as described [243].

3.4. Cellular Transfections Strategies

3.4.1. Transient transfections of HeLa and HEK293T cells using TurboFect

Transfections were performed using cells (e.g. HeLa and HEK 293T) seeded in 6-well tissue culture plates or 60 mm tissue culture dishes (Costar). For 6-well plates, 5 x 10^5 cells were seeded in 2 ml DMEM/10% FBS per well and incubated overnight prior to transfection with 1μg of 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. Samples were gently mixed and incubated for 20 min at room temperature. The DMEM-plasmid-TurboFect solution was then evenly layered dropwise onto the cells, which were then incubated for 24–48 h at 37^{o}C to allow for protein production. Co-transfections with different expression constructs were performed as described for single transfections except 1 μg of each plasmid were both diluted in 190 μl of serum-free DMEM. For transfections in 60 mm dishes, the same procedure for 6-well plates was followed except initial seeding, volume of medium, amount of plasmid, and amount of TurboFect were all doubled. Prior to seeding cells for transfection

experiments, cell viability was examined using Trypan blue staining to ensure that cultures had >95% viable cells prior to seeding.

3.4.2. Transient transfections of HEK293T cells using PEI

Transfections were performed using HEK 293T cells seeded in 6-well tissue culture plates (Costar). Five hundred thousand cells were seeded in 2 mL DMEM/10% FBS per well and incubated overnight prior to transfection with the KIR/IpLITR chimeras and pXM139-Csk. The vector pXM139, which contains the adenovirus major late promoter and the SV40 origin of replication, was generously provided by Dr. Andre Veillette (Institut de recherches cliniques de Montreal [245]. For each expression construct, 1 µg of DNA was first diluted in 190 µl of serum-free DMEM and then 4 µl of PEI in vitro transfection reagent (Sigma–Aldrich) was added. Samples were gently mixed and incubated for 20 min at room temperature. The DMEM-plasmid-PEI solution was then evenly layered onto the cells, which were then incubated for 48 h at 37°C to allow for protein production. Co-transfections with different expression constructs were performed as described for single transfections except 1 µg of each plasmid were diluted in 190 µl of serum-free DMEM. Prior to seeding cells for transfection experiments, cell viability was examined using Trypan blue staining to ensure that cultures had >95% viable cells prior to seeding.

3.4.3. Retroviral transduction of YTS cells

YTS cells were transduced with KIR/LITR1.1b, KIR/LITR 1.1b TR, or KIR/LITR2DL3 constructs in the vector pMX-puro using the Phoenix Helper-dependent protocol as described:

(http://www.stanford.edu/group/nolan/protocols/pro_helper_dep.html). Briefly, 1.5 x 10^6 Phoenix cells were plated in a 6 cm plate (Becton Dickenson). Five to ten μg of DNA are added to 438 μL of H_2O and 61 μL $CaCl_2$. This solution is mixed with 500 μL of 2X HEPES buffered saline (HBS). This solution was then added dropwise onto media. After 48 h, the cellular supernatant was collected and centrifuged for 1500 rpm for 5 minutes. 5 X 10^5 YTS cells were pelleted and resuspended in 1 mL of Phoenix supernatant and 1 μL 1000X polybrene. After 24h, viral supernatant was replaced with fresh YTS media. After 24h, this YTS media was replaced with fresh YTS media supplemented with 1 μg/ml puromycin. Sub-clones were isolated by sorting (FACSAria; BD Biosciences) with the anti-KIR2D antibody DX27 and selected for high receptor expression. YTS cells transduced with the KIR2DL1/SHP-1 WT and KIR2DL1/SHP-1 DA, which contains a mutation in the catalytic phosphatase region resulting in a non functional enzymatic activity, were made previously and a gift from Dr. Deborah Burshtyn [73].

3.4.4. Infection with recombinant vaccinia virus

NK92 or mouse NK cells were washed into Iscove's media supplemented with 2mM L-glutamine, 1X non-essential amino acids, 0.2% BSA and 100 U/ml rIL-2. The cells were infected at a multiplicity of infection (MOI) of 20 with vaccinia virus at 37^{o}C with 5% CO_2. Cells were used after 4 hours of infection. All experiments involving vaccinia virus infection were carried out in the presence of Cytosine B-D-arabinofuranoside-HCl (Ara-C; Sigma Aldrich) at a final concentration of 40 μg/ml to prevent replication of viral DNA by inhibiting deoxycytidine use [246].

3.5. Flow cytometry and fluorescence microscopy

Flow cytometry for the detection of KIR/IpLITR and HA- tagged 'native' IpLITR surface expression was performed using transfected HEK 293T cells. After 48 h post-transfection, the DMEM/transfection solution was removed from each well and the cells were gently washed with 2 ml of sterile Dulbecco's PBS (D-PBS) containing 2 mM of EDTA. PBS was removed and 500 µl of 0.25% Trypsin-EDTA (Gibco) was added to each well and the plate gently agitated until cells detached. Two milliliters of DMEM-10% FBS was added and aliquots of 2 x 10^5 cells placed in 1.5 ml Eppendorf tubes. Cells were centrifuged at 4^{o}C (400 x 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) added. Prior to addition of primary antibody, cells were centrifuged once more and ASB aspirated. Cell pellets were gently disrupted and 1 µg of primary antibody (i.e. anti-DX27 mAb or anti-HA mAb clone HA.C5) diluted in 50 µl of sterile FACS buffer was added. Cells were incubated on ice for 30 min with gentle mixing every 10 min followed by the addition of 1.2 ml ice-cold ASB, centrifugation, and aspiration. Cells pellets were again disrupted and 0.5 µg of goat anti-mouse IgG (H + L)-PE diluted in 50 µL of FACS buffer was added and staining/washing repeated as above. Cells were then analyzed for fluorescence using a FACSCalibur flow cytometer (Becton Dickinson). For flow cytometry to measure KIR/IpLITR surface expression on recombinant vaccinia virus infected NK92 cells or NK cells, staining was performed 4 hours post infection and all data collection was conducted using the Level 2 FACS Canto in the Department of Medical Microbiology at the University of Alberta. For YTS cells, staining was performed on the third day post selection.

To visualize cell surface expression of KIR/IpLITR constructs and HA-tagged 'native' IpLITR, HeLa cells were seeded at 5 x 10^4 cells in 35 mm glass bottom dishes (MatTek), transfected and then antibody stained as described above for flow cytometry. After the final wash, cells were fixed with formalin for 10 min at room temperature, rinsed with D-PBS and then analyzed using a Zeiss Axiovert 2500 M fluorescence microscope.

3.6. Immunoprecipitation and Western blotting

Forty-eight hours after transfection, HEK 293T cells (1 x 10^6) were washed with 1 ml D-PBS/EDTA then treated with 500 µl D-PBS containing 10 µM H_2O_2 and 0.1 µM sodium pervanadate (Na_3VO_4) for 15 min at 37^{o}C. Pervanadate solution was then aspirated following centrifugation of the cells at 4^{o}C. Cell pellets were lysed for 2 hours with 500 µl of ice-cold immunoprecipitation (IP) buffer (50 mM Tris–HCl, 150 mM NaCl, 1% Triton X- 100, supplemented with complete mini EDTA-free protease inhibitor and phosphatase inhibitor cocktail tablets; Roche Diagnonstics). Following the removal of cellular debris by centrifugation at 16,000 x g, cellular lysates were incubated with 10 µg of either anti-KIR mAb (DX27), 1 µg of anti-HA mAb clone HA.C5, or 1µg of anti-FLAG M2 mAb for 2 h at 4^{o}C on a rotary mixer. Fifty microliters of pre-washed protein G sepharose beads (GE Healthcare), resuspended in a 50% slurry with IP lysis buffer, were then added to the samples and incubated for a further 2 h at 4^{o}C on a rotary mixer. Beads were washed three times with 1 mL of IP buffer followed by the addition of 100 µl of 2X SDS-PAGE reducing buffer. Samples were then boiled for 10 min at 100^{o}C and stored at -20^{o}C prior to analysis.

Twenty microliters samples (equivalent to ~0.25 x 10^6 lysed cells) were electrophoresed on 10% SDS-PAGE gels, 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. Nitrocellulose membranes were incubated in Tris-buffered saline supplemented with 0.1% Tween 20 and 5% skim milk (TTBS-SKIM) for 30 min at room temperature. Membranes were incubated overnight at 4°C with anti-phosphotyrosine mAb, anti-KIR mAb (LIG-1), anti-HA-HRP, anti-FLAG-HRP, anti-Csk pAb, or anti-SHP-1-HRP in TBS supplemented with 0.1% Tween 20% and 5% skim milk (TTBS-SKIM) for the detection of immunoprecipitated proteins. If the primary antibody was not conjugated with HRP, the membranes were washed 3 x with TTBS and incubated for 1 h at room temperature with 1:5000 (v/v) of goat anti-mouse IgG (H + L)-HRP diluted in TTBS-SKIM for detection of anti-KIR mAb (LIG-1) reactive proteins or 1:5000 (v/v) of streptavidin-HRP for detection of anti-phosphotyrosine-biotin reactive proteins. After a final wash in TTBS, immunoreactive bands were detected using the SuperSignal West Pico Chemiluminescent Substrate kit (Pierce Biotechnology).

Forty-eight hours post-transfection, HEK 293T cells were washed with 1 ml D-PBS/EDTA, harvested with 0.25% trypsin, and then stimulated with 1 mL D-PBS containing 10 μM H_2O_2 and 0.1 μM sodium pervanadate (Na_3VO_4) for 10 min at 37°C. Pervanadate solution was then aspirated following centrifugation (400 x g, 6 min) of the cells at 4°C. Cell pellets were then lysed with 500 μl of ice-cold immunoprecipitation (IP) buffer (50 mM Tris–HCl, 150 mM NaCl, 1% Triton X-100, pH 7.4, supplemented with complete mini EDTA-free protease inhibitor and phosphatase inhibitor cocktail

tablets; Roche Diagnostics). Following the removal of cellular debris by centrifugation at 16,000 x g, cellular lysates were incubated with 2 µg of anti-HA mAb clone HA.C5 for 14–16 h at 4°C on a rotary mixer. Fifty microlitres of pre-washed (with IP buffer) protein G Sepharose beads (GE Healthcare) were then added to the samples and incubated for a further 2 h at 4°C on a rotary mixer. Beads were washed three times with 200 µl of IP buffer followed by the addition of 60 µl of 2x SDS–PAGE reducing buffer. Samples were then boiled for 10 min at 100°C and stored at -20°C prior to analysis. Twenty microliters of the samples were electrophoresed on 8% SDS–PAGE gels, transferred to 0.2 µm nitrocellulose membranes (Bio-Rad) and then stained with Ponceau S (Sigma–Aldrich) to ensure successful transfer and equivalent loading of samples. Blots were then blocked in TTBS-SKIM for 30 min at room temperature. Membranes were then incubated 14–16 h at 4°C with anti-HA-HRP mAb or anti-CSK antisera diluted 1:1000 v/v in TTBS-SKIM for the detection of immunoprecipitated proteins. For blots incubated with anti-CSK antisera, the membranes were washed 3 times with TTBS and incubated for 1 h at room temperature with 1:5000 (v/v) of goat anti-rabbit IgG (H + L)-HRP. After three washes with TBS, immunoreactive bands were detected using the SuperSignal West Pico Chemiluminescent Substrate kit (Pierce Biotechnology).

3.7. Identification of zebrafish SHP-1 and SHP-2

Zebrafish SHP-1 and SHP-2 cDNAs were identified by tBLASTn searches of the zebrafish database (http://compbio.dfci.harvar- d.edu/tgi/cgi-bin/tgi/Blast/index.cgi) using human SHP-1 and SHP- 2 protein sequences as queries (NCBI accession numbers: AAA36610 and BAA02740) and default settings. The top match for human SHP-1 was zebrafish TC304017, which was then translated into a protein sequence using the

ExPASy Proteomics Sever Translate Tool (http://ca.expasy.org/). This predicted protein was then used to search NCBI's non-redundant database for a match resulting in the identification of a 589 amino acid protein (NCBI accession number NP956254). A similar strategy was used to search for zebrafish SHP-2, leading to the identification of zebrafish TC304103 from the tBLASTn search, which was then translated and used to search NCBI's non-redundant database to identify the top match that was a 594 amino acid protein (NCBI accession number NP956140). Predicted protein structure of the zebrafish phosphatases and identification of the SH2-domains and protein tyrosine phosphatase catalytic (PTPc) domains were also obtained from BLAST analyses and amino acid alignments were performed using the MegAlign program of the DNASTAR Lasergene 7 software.

3.8. Expression analyses of zebrafish SHP-1 and SHP-2

Zebrafish embryos collected after 2 h, 12 h, 24 h, 48 h, and 72 h post-fertilization (hpf) were pooled for each time point and total RNA was isolated using TriZol reagent (Invitrogen) according to manufacturer's instructions. Tissues (kidney, spleen, liver, and gill) were also removed from 10 individual zebrafish and each organ was pooled together and total RNA isolated. Expression analysis of ZfSHP-1 and ZfSHP-2 mRNA was then performed by RT- PCR using freshly isolated RNA (1 μg) converted into cDNA using Oligo(dT)18 primer and 40U of M-MuLV reverse transciptase (Fermentas). Amplification was then performed in 20 ml reactions with 0.5 U Taq DNA polymerase (Fermentas) and 1 mM of primers specific for ZfSHP-1 and ZfSHP-2 (Table 3-1) according to manufacturer's instructions. Cycling parameters were as follows; 2 min and 30 s at 94^{o}C, 35 cycles of 94^{o}C for 30 s, 64^{o}C for 30 s, 72^{o}C for 30 s, and a final

extension step for 7 min at 72 ^{o}C. Reactions were separated on a 1.0% TAE-agarose gel, and visualized by staining with ethidium bromide solution (50 μg/ml).

To generate C-terminal FLAG-tagged ZfSHP-1 and ZfSHP-2, the full-length cDNAs were cloned into the p3XFLAG-CMV-14 expression vector (Sigma–Aldrich). Zebrafish kidney cDNA was amplified with ZfSHP p3XFLAG EcoRI/Fwd and ZfSHP p3XFLAG XbaI/Rvs primers using the same cycling parameters for ZfSHP expression analysis. The PCR products were then excised, purified and subcloned into pJET1.2/blunt, then restriction digested with EcoRI and XbaI and the inserts T4 ligase-mediated cloned into the p3XFLAG-CMV-14 expression vector and the constructs sequenced prior to use in transfection studies.

3.9. Expression of zebrafish Csk

Zebrafish embryos collected after 72 h post-fertilization were pooled and total RNA was isolated using TriZol reagent (Invitrogen) according to manufacturer's instructions. Expression analysis of Csk mRNA was then performed by RT- PCR using freshly isolated RNA (1 μg) converted into cDNA using Oligo(dT)18 primer and 40U of M-MuLV reverse transciptase (Fermentas). Amplification was then performed in 20 ml reactions with 0.5 U Taq DNA polymerase (Fermentas) and 1 μM of primers specific for Csk (Table 3-1) according to manufacturer's instructions. Cycling parameters were as follows; 2 min and 30 s at 94^{o}C, 35 cycles of 94^{o}C for 30 s, 64^{o}C for 30 s, 72^{o}C for 30 s, and a final extension step for 7 min at 72 ^{o}C. Reactions were separated on a 1.0% TAE-agarose gel, and visualized by staining with ethidium bromide solution (50 μg/ml).

3.10. Infection of NK cells with recombinant vaccinia virus

Primary mouse NK cells were washed into Iscove's media supplemented with 2mM L-glutamine, 1X non-essential amino acids, 0.2% BSA and 100 U/ml rIL-2. The cells were infected at a multiplicity of infection (MOI) of 20 with vaccinia virus at 37^{o}C with 5% CO_2. All experiments involving vaccinia virus infection were carried out in the presence of Ara-C (Sigma) at a final concentration of 40 μg/ml to prevent replication of viral DNA.

3.11. Cytolysis assay with vaccinia virus

After virus infection, mouse NK cells or NK92 cells were washed, counted, and diluted to the appropriate concentrations in warm assay medium (Iscove's media with 5% FBS and 2mM L-glutamine) with 100 U/mL rIL-2. Ara-C was introduced at 40μg/ml following infection and maintained throughout the experiment. Cytolysis was measured by chromium release as follows: Target cells were labeled with ^{51}Cr-Sodium Chromate (Perkin Elmer), washed three times in warm assay media, diluted to the appropriate concentration ($2.5x10^5$ cells/ml), plated with NK cells in triplicate and incubated at 37^{o}C with 5% CO_2 for four hours. For the antibody blocking experiments, NK cells were pre-incubated in twice the final concentration of blocking antibody (DX27) for 20 minutes at room temperature, and then mixed 1:1 with target cells. Chromium release was quantified for 50μl of supernatant incorporated into 150 μl of scintillation fluid and analyzed in a 1450 Microbeta Trilux (Wallac). ^{51}Cr release was calculated as: % lysis= 100 X (mean sample release – mean spontaneous release)/(mean total release – mean spontaneous release).

3.12. Synthesis of biotinylated IpLITR CYT peptides

The following peptides were synthesized in order to determine the recruitment potential of IpLITR ITIMs: Biotin-GPSDVIYTELEIK-$CONH_2$, Biotin-GPSDVI(pY)TELEIK-$CONH_2$, (where pY represents a phosphotryrosine), Biotin-ALSGAVYAQVMKK-$CONH_2$, and Biotin-ALSGAV(pY)AQVMKK-$CONH_2$. All peptides were purchased from the IBD Core (University of Alberta, Edmonton, Canada) and supplied at a purity of >95%. The pY residue was incorporated during peptide synthesis. The peptides were synthesized by *N*-(9-fluorenyl)methoxycarbonyl (FMoc) chemistry, purified by reversed-phase high pressure liquid chromatography, and analyzed for purity by ion spray mass spectrometry and ^{31}P NMR. Phosphopeptides were dissolved at 0.1mg/mL in Acetonitrile:H_2O (4:1).

3.13. Protein recruitment with synthetic biotinylated IpLITR CYT peptides.

YTS or 3B11 cells (1-5x10^7) were lysed at 1 x 10^6 cells/ml in ice-cold IP buffer (50 mM Tris–HCl, 150 mM NaCl, 1% Triton X-100, pH 7.4, supplemented with complete mini EDTA-free protease inhibitor and phosphatase inhibitor cocktail tablets; Roche). Following the removal of cellular debris by centrifugation at 16,000 x g, cellular lysates were incubated with 100 μg of the biotinylated synthetic IpLITR peptide (i.e. Biotin-GPSDVIYTELEIK-$CONH_2$, Biotin-GPSDVI(pY)TELEIK-$CONH_2$, Biotin-ALSGAVYAQVMKK-$CONH_2$, or Biotin-ALSGAV(pY)AQVMKK-$CONH_2$) and left to rotate overnight (14-16 h) at 4^{o}C. The next day, 500 μl of washed streptavidin-coated sepharose beads (GE Healthcare) were added to the lysate-peptide mixture and the slurry was returned to rock overnight at 4^{o}C. After the incubation was complete, the mixture

was centrifuged at 400 x g for 5 minutes at 4^{o}C. The cell lysate was collected and stored using gel-loading tips and the streptavidin and peptides were washed with 50 ml of IP buffer for 20 minutes rotating at 4^{o}C. After the wash, the mixture was centrifuged at 400 x g for 5 minutes at 4^{o}C. The wash process was repeated 3 times and after it was completed the streptavidin-peptide complex was resuspended in 1ml of 2x SDS-PAGE reducing buffer and boiled for 10 minutes. These samples were then used for Western blotting experiments.

3.14. Statistics

A Student's T-test (two tails) was performed when assessing potential differences between experimental groups. P-values <0.05 were designated as statistically significant.

Table 3.1. Primers used in this thesis.

Primer Name	Primer Sequence 5' to 3'
[1]pSPORT-KIR2DL3TM Rvs	AAGGAGAAAGAAGAGGAGGAGGATGAAGAGGATGATGACC
[2]pSPORT-KpnI Fwd	ATACTAGGTACCTCTAGAGGATCCAAGCTTACGTACGC
[3]CONSTRUCT 1.2a Fwd	P-CGTTGGTGGCACAAATCCAAC
[3]CONSTRUCT 1.2a KpnI/Rvs	ATACTAGGTACCCTATGTGTTCTGCTTCAGCTGTGAGT
[3]CONSTRUCT 1.1b Fwd	P-CACAAATCCAACAAAGGAAAGGAC
[3]CONSTRUCT 1.1b TR KpnI/Rvs	ATACTAGGTACCCTATTTATTCTTGTATGACTCCTT
[3]CONSTRUCT 1.1b KpnI/Rvs	ATACTAGGTACCCTATGTGTTCTGCTTCAGCTGTGA
[4]KIR_{ED}-$LITR_{CYT}$ pBABE SalI/Fwd	ATACTAgtcgacgcaccATGTCGCTCATGGTCGTCAGCA
[4]KIR_{ED}-$LITR_{CYT}$ pBABE NotI/Rvs	ATACTAgcggccgcCGCGTACGTAAGCTTGGATCCTCT
[5]pDISPLAY L1.1b/SmaI Fwd	CCCGGGGTTCTGTCTGTGGAGCCG
[5]pDISPLAY L1.1b/SalI Rvs	GTCGACCTATGTGTTCTGCTTCAGCTG
[6]pJET1.2 Fwd sequencing	CGACTCACTATAGGGAGAGCGGC
[6]pJET1.2 Rvs sequencing	AAGAACATCGATTTTCCATGGCAG
[7]ZfSHP-1 Fwd expression	CCTGTATGGTGGGGAGAAGTTTGCC
[7]ZfSHP-1 Rvs expression	ACGGTGTAGCGGTCATTATTGC
[7]ZfSHP-2 Fwd expression	CAGCGAAGGCAAACCCAAAGTCACA
[7]ZfSHP-2 Rvs expression	TGATCAGGCCAGGCACGGAAATG
[8]ZfSHP-1 p3XFLAG EcoRI/Fwd	GAATTCATGGTTCGGTGGTTTCACAGA
[8]ZfSHP-1 p3XFLAG XbaI/Rvs	TCTAGATCGTTTCCTAACAGATCC
[8]ZfSHP-2 p3XFLAG EcoRI/Fwd	GAATTCATGACATCCCGAAGGTGGT
[8]ZfSHP-2 p3XFLAG XbaI/Rvs	TCTAGATCTGTGACTCTTCTGCTGCTG
[9]KIR/IpLITR1.1b TR Y1F Fwd	CTTCAGACGGATGAGCACATTTTTGACACTGTGGA
[9]KIR/IpLITR1.1b TR Y1F Rvs	TCCACAGTGTCAAAAATGTGCTCATCGGTCTGAAG
[9]KIR/IPLITR1.1b TR Y2F Fwd	GAACTCAGTGGGGCCGTTTTTGCACAGGTCAT
[9]KIR/IpLITR1.1b TR Y2F Rvs	ATGACCTGTGCAAAAACGGCCCCACTGAGTTC
[10]ZfCsk Fwd expression	TAGGTTTTGGGCCAGTTTTG
[10]ZfCsk Rvs expression	CCCATGGAGGTGTTTGTACTC
[11]ZfCsk p3XFLAG NotI/Fwd	GCGGCCGCTAGGTTTTGGGCCAGTTTT
[11]ZfCsk p3XFLAG BamHI/Rvs	GGATCCCCCATGGAGGTGTTTGTACTC

Table 3.1. Cont'd

Notes:

[1]Primer used for amplification in the reverse direction around the pSPORT-1/KIR2DL3 plasmid starting immediately after the TM sequence of KIR2DL3 (i.e. bp position 795).

[2]Primer used for the amplification in the forward direction around the pSPORT-1/KIR2DL3 plasmid and introduction of a KpnI site immediately after the TM sequence of KIR2DL3 (i.e. bp position 795).

[3]Amplification of the CYT regions of LITRs for cloning into the pSPORT-1/KIR2DL3 plasmid prepared with [1]pSPORT-KIR2DL3TM Rvs and [2]pSPORT-KpnI Fwd.

[4]Amplification of KIR_{ED}-$LITR_{TM}$ from pSPORT-1 and cloning into pBABE.

[5]Amplification of TS32.17 L1.1b (NCBI accession number: ABI16050) from pCR4-TOPO and cloning into pDISPLAY.

[6]Colony PCR primers.

[7]RT-PCR primers

[8]Amplification of Zebrafish SHP-1 / SHP-2 and cloning into p3XFLAG-CMV-14.

[9] Site-directed mutagenesis of KIR/IpLITR1.1b TR cytoplasmic tyrosines to phenylalanines.

[10]RT-PCR primers

[11]Amplification of Zebrafish Csk

CHAPTER IV

BIOCHEMICAL CHARACTERIZATION OF PUTATIVE INHIBITORY CHANNEL CATFISH LEUKOCYTE IMMUNE-TYPE RECEPTORS, IpLITR1.2a AND IpLITR1.1b.

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4.1. Introduction

Immunoregulatory IgSF members often share a common mode of intracellular signaling through distinct stimulatory and inhibitory pathways [26, 30, 35]. These features are constant for several mammalian innate immune receptors and are likely conserved in non-mammalian vertebrates [2, 145, 149, 183]. In general, stimulatory innate immune receptors have CYT tails lacking any signaling motifs but encode a positively charged TM region that facilitates association with the negatively charged TM region of an adaptor molecule, which contains a longer CYT tail encoding ITAMs [226, 247, 248]. In contrast, inhibitory receptors encode their own long CYT tail that contains one or more ITIMs that, upon receptor engagement, are phosphorylated at tyrosine residues by SFKs [53, 249]. The phosphorylated tyrosine(s), within the context of an ITIM, subsequently recruit SHP-1, SHP-2, or SHIP, which dephosphorylate tyrosines on

signaling molecules involved in effector responses thus abrogating kinase-dependent cellular immune responses [50, 52, 73, 250].

Although the majority of innate immune receptor research has focused on mammalian immunoregulatory receptor families, recent work has identified the existence of numerous families of IgSF-like genes in non-mammalian vertebrate species such as birds, amphibians, and fish [2, 127, 148, 149, 188, 209, 212, 251]. However, many of these 'novel' IgSF receptors remain to be functionally characterized regarding their signaling potential and ultimately their contribution to the control and coordination of immune cell effector functions.

The channel catfish is currently the best-characterized immune model in fish as it is the only teleost to have available long-term leukocyte cell lines including T cells, B cells, macrophages, and NK-like cells [166, 169, 252-255]. These cell lines serve as an integral tool for characterization of teleost immunity and uncovering aspects of the evolution of vertebrate immunity. For example, channel catfish NK-like recognize allogeneic targets (e.g. irradiated B cells) and kill them in a calcium-dependent fashion [170]. Presently, the receptors responsible for recognition and regulation of these cytotoxic responses are unknown. A recently identified family of receptor proteins, termed IpLITRs, have characteristics that suggest these may, in part, be candidates for this regulation [2]. These polymorphic proteins belong to the IgSF and consist of both inhibitory and stimulatory forms co-expressed in hematopoietic tissues as well as various catfish myeloid and lymphoid cell lines [2]. IpLITR mRNA levels were also preferentially upregulated in cytotoxic lymphocytes following alloantigen stimulation, but not LPS stimulation, and detailed sequence analysis of the expressed cDNA indicated

an unexpectedly large array of co-expressed putative inhibitory and stimulatory forms [3].

It has already been established that IpLITRs that contain a charged residue in the TM region associate with the channel catfish ITAM-containing adaptor molecules IpFcRγ and IpFcRγ-L [156]. Antibody cross-linking of an HA epitope-tagged chimeric receptor consisting of the IpLITR extracellular region fused to the ITAM bearing CYT tail of IpFcRγ-L, in the mammalian RBL-2H3 cell line, lead to activation of the ERK pathway [157]. These results are consistent with those of the zebrafish IgSF receptor NITR9, which was found to associate with DAP12 and signal through the ERK pathway following Ab cross-linking in the AD 293 cell line [155]. Additionally, this chimeric IpLITR/ IpFcRγ-L receptor induced cellular degranulation and promoted phagocytosis of anti-HA mAb-coated agarose beads providing additional functional information regarding the functional roles of stimulatory IpLITR-types. Conversely, there has been no functional or biochemical characterization of teleost inhibitory receptor-types including ITIM-containing IpLITRs.

My investigations on the potential for IpLITRs to function as inhibitory immunoregulatory receptors focused on two of the putative inhibitory IpLITRs identified from the TS32.17 cell line following alloantigen challenge, known as TS32.17 IpLITR1.2a (accession no. ABI16051) and TS32.17 IpLITR1.1b (accession no. ABI16051) [3]. These receptors share high similarities in their extracellular regions (i.e. both encode four C2-type Ig-like domains) as well as they both have uncharged TM segments. IpLITR1.1b and IpLITR1.2a are differentiated by the fact that IpLITR1.1b contains an additional region of the CYT tail proximal to the TM segment. This region

does not encode any classical ITIMs but does contain three tyrosine residues. Distal from the TM region, both IpLITR1.1b and IpLITR1.2a contain nearly identical regions that contain both canonical ITIMs and an Immunoreceptor Tyrosine-based Switch Motif (ITSM). An amino acid alignment of IpLITR1.1b and IpLITR1.2a with the CYT tyrosine motifs classified as 'itim?', a nonclassical ITIM-like motif; 'ITIM', the canonical ITIM; and 'ITSM' is presented in Figure 4.1. These two receptors were chosen for two reasons: Firstly, to determine if IpLITR1.1b and IpLITR1.2a are bona fide inhibitory immunoregulatory receptors; and secondly, to investigate the difference that this unique proximal CYT region of IpLITR1.1b may provide.

My research hypothesis is that ITIM-bearing IpLITR1.2a and IpLITR1.1b are inhibitory receptors that recruit inhibitory phosphatases, SHP-1 and SHP-2 to the phosphotyrosines within their ITIMs. My specific research objectives for this chapter were; i) to develop chimeric inhibitory receptor expression constructs in order to examine the signaling potential of IpLITR1.2a and IpLITR1.1b, and; ii) to identify teleost SHP-1 and SHP-2 and examine if the IpLITRs recruit these phosphatases.. In this chapter, I present the first biochemical characterization of putative inhibitory forms from the IpLITR family.

4.2. Results

4.2.1. KIR/IpLITR expression constructs

In order to characterize IpLITR1.1b and IpLITR1.2a, I first developed expression constructs in which I fused the EC and TM regions of KIR2DL3 to the CYT regions of IpLITR1.1b and IpLITR1.2a. In addition, I also developed a chimeric receptor that contained only the TM proximal CYT region of 1.1b and this was termed KIR/IpLITR1.1b TR with the TR indicating that it was truncated. KIR/IpLITR1.1b TR contains no canonical ITIMs but it does contain three tyrosine residues and the purpose of this chimera was to determine if any of these non-ITIM residues had any effect on inhibition as determined by the recruitment of inhibitory phosphatases SHP-1 and/or SHP-2. The last construct that was developed contained a stop codon shortly after the TM region and was termed KIR/LITRΔCYT. The purpose of this chimeric construct was to verify that any results were not due to the EC or TM regions of KIR2DL3 and all results were strictly IpLITR CYT-mediated. A schematic representation of the four chimeric KIR/IpLITR constructs is presented in Figure 4.2.

4.2.2. Surface expression of KIR/IpLITR chimeras

Following transfection in HEK 293T cells, the expression of KIR/IpLITR was determined by flow cytometry and microscopy (Fig. 4.3, 4.4). Compared with mock-transfected cells (i.e. transfection reagent alone) constructs 1.2a, 1.1b, 1.1b TR, and ΔCYT exhibited a significant increase in staining (>80%) using the anti-KIR2D mAb (DX27). Cells stained with secondary antibody alone (i.e. goat anti-mouse IgG-PE) had a

similar staining pattern as the mock-transfected cells. To visualize the cell surface staining of each construct, fluorescence microscopy was performed on HeLa cells transfected with each of the constructs followed by staining with the anti-KIR2DL3 (DX27) mAb and discrete KIR/LITR staining localized to the cell surface for all four of the chimeric constructs (Fig. 4.4). No staining was observed with mock-transfected cells, cells stained with secondary antibody alone, or isotype control staining (data not shown).

4.2.3. Phosphorylation of KIR/IpLITR chimeras

Following pervanadate treatment, HEK 293T cell lysates were probed with the anti-KIR2DL3 mAb (DX27) and immunoprecipitated samples were then examined for tyrosine-phosporylation by WB (Fig. 4.5B). Tyrosine phosphorylation was observed following pervanadate treatment of HEK 293T cells that were transiently transfected with KIR/IpLITR1.1b, 1.2a, or 1.1b TR, which exhibited two distinct staining patterns when blotted with a biotinylated anti-phosphotyrosine antibody (Fig. 4.5B). For example, a discrete (~50 kDa; indicated by arrow) as well as a diffuse stained band (~60–70 kDa; indicated by an asterisk) was detected from cells transfected with KIR/IpLITR 1.1b. The same pattern was also observed for KIR/IpLITR 1.1b TR but the relative sizes of the discrete and diffuse staining bands were smaller than those observed for KIR/LITR 1.1b (i.e. ~45 kDa and 55–60 kDa, respectively). KIR/IpLITR1.2a-transfected cells immunoprecipitated with DX27 resulted in the detection of a discrete band at ~37 kDa (indicated by an arrow) and a diffuse staining band of ~40–50 kDa (indicated by an asterisk). In comparison, mock-transfected cells and cells transfected with KIR/IpLITR1.1b ΔCYT, which is devoid of cytoplasmic tyrosines, resulted in no detection of tyrosine-phosphorylated proteins (Fig. 4.5B). Furthermore, cells transfected

with the various constructs without pervanadate treatment then immunoprecipitated with DX27 resulted in no detection of tyrosine-phosphorylated proteins (data not shown). I also blotted a second membrane with an anti-KIR2D mAb (LIG-1) and a similar pattern of staining was observed as with the anti-phosphotyrosine stained blot (Fig 4.5A). KIR/IpLITR1.1b, 1.1b TR, and 1.2a all demonstrated discrete (~50 kDa, ~45 kDa, and ~37.5 kDa, respectively; indicated by arrow) and diffuse staining bands (~60–70 kDa, ~55–60 kDa, and ~40 – 50 kDa, respectively; indicated by an asterisk). For KIR/LITRΔCYT a prominent band (~35 kDa; indicated by arrow) and some diffuse staining (~45 kDa; indicated by an asterisk) were also observed. The diffuse staining for this construct was difficult to resolve due to the prominent cross-reacting protein band (~50kDa) that was detected with the goat anti-mouse IgG-HRP antibody (Fig. 4.5). The larger diffuse staining is predicted to be due to the variable glycosylation of the extracellular region of KIR2DL3 that contains four N-linked glycosylation sites, which has previously been demonstrated by others [256, 257]. PNGase treatment of transfected HEK 293T lysates led to a loss of this larger band in all lanes suggesting the band of increased size is in fact due to glycosylation (data not shown). The smaller more discrete bands likely represent non-glycosylated forms of the KIR/IpLITR chimeric proteins that may or may not be expressed on the cell surface (i.e. these may represent immature glycoforms). For mock-transfected cells, at least five non-specific protein bands were observed.

4.2.4. Development of expression constructs for zebrafish tyrosine phosphatases SHP-1 and SHP-2.

In order to determine the SHP-recruitment capabilities of the different KIR/IpLITR phosphoproteins, I first cloned zebrafish SHP-1 and SHP-2 into expression vectors. Zebrafish SHP-1 (589 amino acids) is located on chromosome 16 and SHP-2 (594 amino acids) is located on chromosome 10. Both phosphatases encode N- and C-terminal SH2-domains as well as a protein tyrosine phosphatase catalytic domain (Fig. 4.6A). Expression analysis revealed that in adult tissues of kidney, spleen, liver, and gill both phosphatases were expressed but in zebrafish embryos, SHP-1 and SHP-2 expression was not observed until 48 h post fertilization (Fig. 4.6B). I then generated C-terminal 3XFLAG fusion proteins transfected in HEK 293T cells and immunoprecipitated the SHP proteins with an anti-FLAG M2 mAb. Following separation of samples on SDS-PAGE and Western blotting, I observed that recombinant zebrafish SHP-1 and SHP-2 were produced as ~65 kDa proteins (Fig. 4.6C). When compared with human and mouse SHP-1 and SHP-2 amino acid sequences, the teleost proteins are ~67% identical (~78% similar) (Fig. 4.7) and ~91% identical (~94% similar) (Fig. 4.8), respectively. The N- and C-terminal SH2-domains are also highly conserved between teleost and mammalian SHPs and the PTP signature motif (HCxAGxGRS/T, where x is any amino acid) is identical between these proteins in mammals and fish (Fig. 4.7, 4.8).

4.2.5. KIR/IpLITR chimeras recruit both SHP-1 and SHP-2 following tyrosine phosphorylation

Individual KIR/IpLITR chimeric constructs (KIR/IpLITR1.1b, 1.1b TR, 1.2a, and ΔCYT) were transiently transfected along with either 3XFLAG zebrafish SHP-1 or 3XFLAG zebrafish SHP-2 in HEK 293T cells for 48 hours. Cells were then collected, washed, and treated with pervanadate, lysed, then immunoprecipitated with the DX27 mAb. Both KIR/IpLITR1.1b and 1.2a recruited SHP-1 (Fig. 4.9A) and SHP-2 (Fig. 4.9B) following pervanadate stimulation. KIR/IpLITR1.1b TR did not recruit SHP-1 but a faint band for SHP-2 was observed that was much weaker than the SHP-2 bands that co-immunoprecipitated with KIR/IpLITR1.1b and KIR/IpLITR1.2a (Fig. 4.9B). This does not appear to be due to lower levels of available SHP-2 in the co-transfected cells since relatively equal amounts of SHP-2 fusion proteins were immunoprecipitated from all samples (Fig. 4.9B). However, there was a noticeably reduced amount of SHP-1 protein that co-immunoprecipitated with KIR/IpLITR1.1b and 1.2a when compared with SHP-2 (Fig. 4.9A). This could in part be due to the reduced amount of zebrafish SHP-1 proteins as observed following immunoprecipitation with anti-FLAG mAb (Fig. 4.9A). Finally, KIR/IpLITRΔCYT, which is devoid of tyrosine residues and does not generate a phosphoprotein following treatment with pervanadate, did not recruit zebrafish SHP-1 nor SHP-2 although both of the fusion proteins were present (Fig. 4.9). In the absence of pervanadate treatment SHP-1 or SHP-2 recruitment was not detected nor were KIR/IpLITR chimeric constructs phosphorylated (data not shown).

4.2.6. Full-length wild-type IpLITR1.1b recruits SHP-1 and SHP-2.

To demonstrate that a non-chimeric construct encoding the extracellular, transmembrane, and cytoplasmic regions of a wild type putative inhibitory IpLITR can be expressed on the cell surface, I generated an N-terminal HA-tagged recombinant fusion protein encoding the TS32.17 L1.1b (NCBI accession number: ABI16050) cDNA fused with the mouse Ig k-chain secretion signal (Fig. 4.10A). This construct encoded four extracellular Ig-like domains, a TM segment, and a CYT region that is identical to KIR/IpLITR1.1b. In addition, no N-linked glycosylation sites are encoded within the EC region of this receptor. While sequencing this construct I discovered that one of the clones had a 'frame-shift' mutation due to the deletion of a G base pair at position 566 (position 1–3 are the start ATG of the mouse Ig k-chain secretion signal encoded by the pDISPLAY vector). This resulted in a truncated receptor due to an in-frame STOP codon (i.e. TGA) at amino acid position 216 located at the C-terminal end of the second Ig-like domain (Fig. 4.10A). This truncated receptor was therefore missing Ig-like domains D3 and D4, the TM segment, and the entire CYT region. I designated this as IpLITR1.1bΔ. This construct was used as a negative control for the expression, phosphorylation, and SHP-1/-2 recruitment experiments.

Following transient transfection in HEK 293T cells, the expression of epitope-tagged IpLITR1.1b was determined by flow cytometry and microscopy (Fig. 4.10B). Compared with mock-transfected cells (i.e. transfection reagent alone) or cells transfected with IpLITR1.1bΔ, a significant increase in anti-HA mAb staining (>70%) was observed when the cells were transfected with IpLITR1.1b (Fig. 4.10B). Cells stained with secondary antibody alone (i.e. goat anti-mouse IgG-PE) or with an isotype control for the

anti- HA mAb (IgG3) followed by secondary antibody staining had a similar staining pattern as the mock-transfected cells (data not shown). To visualize the cell surface staining of TS32.17 L1.1b (IpLITR1.1b), microscopy was also performed followed by staining with an anti-HA mAb. Cells transfected with IpLITR1.1b demonstrated a discrete pattern of cell surface staining (Fig. 4.10B), which was not observed with mock-transfected cells, cells stained with secondary antibody alone, isotype control stained cells, and cells transfected with IpLITR1.1bΔ (data not shown).

HEK 293T cells transiently transfected with either IpLITR1.1b or IpLITR1.1bΔ were then co-transfected with 3XFLAG zebrafish SHP-1 or 3XFLAG zebrafish SHP-2 for 48 h, treated with pervanadate, and immunoprecipitated with the anti-HA mAb (HA.C5). Blots were then probed with an anti-phosphotyrosine mAb (to detect the phosphorylated LITR) (Fig 4.11) or an anti-FLAG HRP-conjugated mAb to determine if zebrafish SHP-1 and/or SHP-2 bound to the phosphotyrosines with the CYT of TS32.17 L1.1b (Fig 4.12). Lysates immunoprecipitated with anti-HA mAb from cells transfected with IpLITR1.1b or IpLITR1.1bΔ after pervanadate treatment showed that only the full-length IpLITR1.1b receptor was phosphorylated as no bands at the size of IpLITR1.1bΔ were detected (Fig 4.11). Co-transfection of IpLITR1.1b with either FLAG-SHP-1 or FLAG-SHP-2 followed by IP and WB resulted in the detection of a phosphoprotein that co-immunoprecipitated with zebrafish SHP-1 and zebrafish SHP- 2 (Fig. 4.12). Conversely, IP of the truncated receptor (IpLITR1.1bΔ) did not result in the detection of zebrafish SHP-1 or SHP-2 (Fig. 4.12). Zebrafish SHP-1 and SHP-2 fusion proteins were produced in all transfected cells, which was verified by IP with the anti-FLAG M2 mAb (Fig. 4.12). Similar to Figure 4.9, the relative amounts of zebrafish SHP-1 fusion protein

was lower than SHP-2 (Fig. 4.12).

4.3. Discussion

Immune cell functions depend in part on the net outcome of opposing signals delivered by stimulatory and inhibitory immune receptors. The importance of this balance can be appreciated in the context of many immune-related diseases. The loss of an inhibitory signal is often associated with unchecked inflammatory responses resulting in a state of autoimmunity [29]. Human genes located within the LRC (e.g. KIRs and LILRs) and on chromosome 1 (e.g. FcRs) have been directly linked with susceptibilities to infectious diseases and autoimmunity [135, 258-264]. The identification of these disease susceptibility genes provides a better understanding of the etiology and pathogenesis of autoimmune diseases and provides clues for the development of immunotherapeutic strategies [27, 265]. Recent studies in birds, amphibians and bony fish indicate that FcR- and LRC-like proteins are conserved across vertebrates [2, 127, 129, 143, 145, 209]. This provides a unique opportunity to functionally characterize innate immunoregulatory receptor networks that will not only provide information regarding the immune responses of non-mammalian animals but will also serve as important model systems for gaining insights into conserved mechanisms of innate immune receptor-mediated regulation of vertebrate immunity.

In this chapter, I present the first biochemical characterization of putative inhibitory forms from the IpLITR family. This was accomplished by generating chimeric receptor constructs, transfecting mammalian cells, and performing co-immunoprecipitations. Chimeric receptors were necessary due to the lack of reagents, specifically anti-IpLITR antibodies, in channel catfish and fish immunology as a whole.

In addition, the cognate ligand for IpLITR1.1b or IpLITR1.2a remains unknown. Thus by fusing the EC and TM regions of KIR2DL3 to the CYT of LITR, I developed chimeric receptors in which the ligand is known (HLA-Cw3), and monoclonal antibodies are available (DX27, LIG-1) to aid me in my studies. In addition, these chimeras gave me the opportunity to later transfect these receptors into mammalian lymphocytes for further functional studies, which will be discussed in the subsequent chapters of this thesis [80, 90].

Characterization of the constructs was performed using transient expression experiments in HEK 293T and HeLa cells in order to examine their surface expression, tyrosine phosphorylation, and phosphatase recruitment potential. All constructs with tyrosine-containing IpLITR CYT regions were expressed on the cell surface and these proteins became phosphorylated following pervanadate treatment. This was not observed in mock-transfected control cells or cells transfected with KIR/IpLITR ΔCYT. These findings demonstrate that putative inhibitory catfish IpLITR CYT tails are specifically phosphorylated at tyrosine residues, which may facilitate their association with phosphatases required for cellular inhibition. Following immunoprecipitation with the DX27 mAb, two major patterns of staining were observed for all chimeric constructs that likely represent glycosylated and immature glycoforms of the proteins. This pattern was detected when blots were probed with either an anti-phosphotyrosine mAb or a mAb specific for KIR2DL3 and both receptor forms (i.e. glycosylated and non-glycosylated) appeared to be tyrosine phosphorylated following pervanadate stimulation. Cell surface expression of the epitope-tagged ‘native’ receptor IpLITR1.1b, encoding four extracellular Ig-like domains, a TM segment, and a long tyrosine-containing CYT region,

was also demonstrated in this study reinforcing that IpLITRs localize to the cell surface, a trait that is shared with the majority of characterized vertebrate innate immunoregulatory receptors. IP of this receptor with the anti-HA mAb from pervanadate-treated cells resulted in the detection of a phosphoprotein (slightly lower than 70kDa) that corresponds to the predicted size of TS32.17 L1.1b. A mutated version of this receptor (i.e. missing the TM and CYT) was not expressed on the surface and was not phosphorylated.

After confirming that each construct was expressed on the cell surface and tyrosine phosphorylated, I predicted if they would bind inhibitory phosphatases (i.e. SHP-1 and SHP-2) based on known mammalian SHP-recruitment motifs [266]. The KIR/IpLITR 1.1b, 1.1b TR, 1.2b, and ΔCYT receptors encoded 2, 3, 6, and 0 cytoplasmic tyrosine residues, respectively. Using the consensus sequence (T/V/ I/Y)-X-pY-(A/S/T/V)-X-(I/V/L) [266], where X represents any amino acid and pY is the phosphotyrosine residue at position 0 (p0), I predicted if the pY within each IpLITR CYT could bind the SH2- domains of the phosphatases SHP-1 and SHP-2. I then examined residues at positions pY +4 to +6 for the large hydrophobic residues W, Y, M, and F and/or the positively charged residues R, K, and H that greatly enhance pY binding to the SH2-domains of SHP-2 and SHP-1, respectively [266]. According to this analysis, all constructs with the exception of KIR/IpLITR ΔCYT are predicted to recruit both SHP-1 and SHP-2 (Fig. 4.13). However, not every tyrosine residue is predicted to contribute to SHP-binding following phosphorylation. Specifically, for IpLITR1.2a, the TS32.17 CYT Y^1 has SHP-1 and SHP- 2 recruitment potential and TS32.17 CYT Y^2 is predicted to only bind SHP-1 (Fig 4.13). The truncated TS32.17 L1.1b CYT (LITR1.1b TR) contains three tyrosine residues but only Y^2 is predicted to bind SHP-1 and SHP-2. Residues Y^1 to Y^3 of

KIR/IpLITR1.1b (containing the full-length CYT of TS32.17 L1.1b) are identical to that of KIR/LITR1.1b TR but this CYT has an additional three tyrosines labeled Y^4, Y^5, and Y^6 (Fig. 4.13). All of these membrane distal tyrosines are predicted to bind SH2-domains. For example, both Y^4 and Y^6 most likely bind SHP-1, whereas Y^5 likely interacts with SHP-2. Finally, KIR/LITR ΔCYT is devoid of any tyrosine residues within its CYT region and is not predicted to interact with SH2-domains and consequently should not bind either phosphatase.

Although SHP-1 and SHP-2 are conserved among endothermic vertebrates (i.e. mammals and birds), invertebrates only have a single SHP-2 orthologue termed Corkscrew (Csw) [259]. A recent study reported a functional relationship between Csw and the *D. melanogaster* engulfment receptor splice variant Draper II [267]. Draper function can be segregated according to its splice variants in which the splice variant Draper I promotes the engulfment of axonal debris through an ITAM while Draper II contains an ITIM. Draper II suppresses Draper I signaling through an ITIM-mediated pathway that involves the recruitment of Csw [267]. This is evidence that the cellular strategy of utilizing tyrosine phosphatases by recruitment to ITIM-like motifs is conserved although some of the specific protein homologs differ for vertebrates and invertebrates.

One of the goals of the work in this chapter was to determine whether there were SHP-1 and SHP-2 homologues in fish and if the ITIM motifs in putative inhibitory IpLITRs are capable of recruiting these proteins, which would be strong evidence towards supporting a role of IpLITRs in the initiation of inhibitory signaling pathways. I identified and cloned zebrafish SHP-1 and SHP-2 cDNAs from the genomic database and

generated C-terminal 3XFLAG-tagged fusion proteins. As in mammals, zebrafish SHP-1 and SHP-2 encode N- and C-terminal SH2-domains required for binding phosphorylated tyrosines and a protein tyrosine phosphatase (PTP) catalytic domain with the PTP signature motif (HC-X-AG-X-GRS/T) [266, 268]. I used zebrafish SHP-1 and SHP-2 in these studies due to the availability of their full-length sequences in the genomic database, high amino acid homology with their mammalian counterparts, and conserved structural features for phosphotyrosine binding and phosphatase activity. I have also searched for the catfish SHP-1 and SHP-2 cDNAs and to date obtained a partial 897 bp fragment for SHP-1 that encodes the PTP portion of the protein.

In co-transfection experiments, both zebrafish SHP-1 and SHP-2 associated with the phosphorylated CYT regions of KIR/IpLITR 1.1b and 1.2a. Compared with KIR/IpLITR1.1b TR, SHP-1 and SHP-2-binding was more prominent for KIR/IpLITR1.1b and KIR/IpLITR1.2a following tyrosine phosphorylation, which contain cytoplasmic tyrosines embedded within ITIM-like motifs (i.e. Y^1 and Y^2 for KIR/IpLITR1.2a and Y^4, Y^5, and Y^6 for KIR/IpLITR1.1b). Conversely, KIR/LITR1.1b TR has three tyrosine residues and none of them are found within a consensus ITIM or ITIM-like motif. However, at the pY^2 +1 to +5 positions of KIR/IpLITR1.1b TR there are residues that are predicted to facilitate SHP- binding (Fig. 4.13). In fact, a weak-staining band for SHP-2 was detected following co-immunoprecipitation with phosphorylated KIR/LITR1.1b TR. From the data collected in this chapter, I predicted that, although all of the tyrosine residues within the various IpLITR CYT are likely phosphorylated following pervanadate treatment, only those found within KIR/LITR1.2a and the C-terminal regions of KIR/LITR1.1b (i.e. Y^4, Y^5, and Y^6) are responsible for strong SHP-

binding. In some cases when I observed zebrafish SHP-1 and SHP-2 binding to the phosphorylated constructs an additional band (~72 kDa) was also detected. This protein may represent a phosphorylated form of the recruited zebrafish phosphatases, which is yet to be confirmed. Finally, co-immunoprecipitation of the epitope-tagged IpLITR TS32.17 L1.1b also demonstrated SHP-1 and SHP-2 binding following tyrosine phosphorylation. Since this construct encoded the same CYT region as KIR/IpLITR1.1b, I also predicted that the C- terminal tyrosines (i.e. Y^4 to Y^6) of this receptor are primarily responsible for the phosphatase recruitment. A truncated version of this protein was not expressed on the surface, did not generate a phosphorylated protein, and did recruit neither SHP-1 nor SHP-2. Therefore, the results confirmed that, like mammalian inhibitory ITIM-containing receptors, IpLITRs recruit SHP-1 and SHP-2 as an initial step required for inhibitory immune receptor-mediated signaling.

In mammals, SHP-1 and SHP-2 have been identified as key mediators involved in the control of cellular responses. Activation of these phosphatases modulate the outcomes of many cellular processes including: growth and development, inflammatory responses, apoptosis, and a wide range of intracellular signaling cascades (reviewed in [269]). Specifically, SHP phosphatases can diminish Toll-like receptor-mediated inflammatory cytokine production [270], inhibit B cell receptor-induced cellular activation [271], inhibit the proliferation, cytotoxicity, and cytokine production of T cells [272], and are key mediators of the inhibitory signals transmitted by KIRs [51, 52]. In addition, intracellular parasites such as *Leishmania donovani* can activate host cell SHP-1 as a survival strategy in order to inhibit the generation of nitric oxide production [273] and SHP manipulation by certain cancers results in the promotion of tumor growth and

survival [274-276] reinforcing that these signaling molecules are essential components of efficient immune responses and healthy cell processes.

Although there are many examples of putative inhibitory immunoregulatory receptors in teleost fish, this is the first example of two such receptors recruiting SHP-1 and SHP-2 following tyrosine phosphorylation. This is an important first step in the induction of an inhibitory signaling cascade but to further understand their influence(s) on leukocyte effector functions, additional studies are required. However, these results set the stage for exploring the functional consequences of phosphatase recruitment by teleost innate immune receptors belonging to the IgSF family and further studies to characterize these IpLITR-mediated signaling events continue in the proceeding chapters.

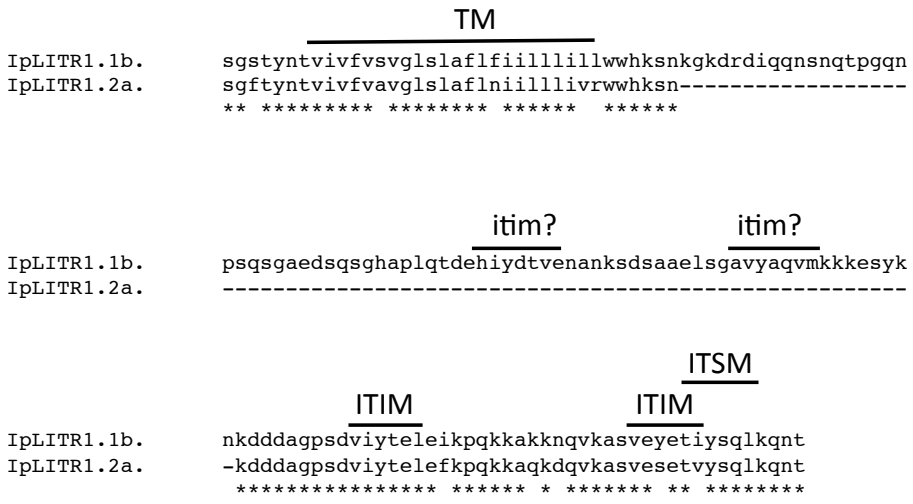

Figure 4.1. Amino acid sequence alignment of the transmembrane and cytoplasmic regions of TS32.17 IpLITR1.1b (accession number: ABI10650) and TS32.17IpLITR1.2a (accession number: ABI10651). IpLITR1.1b and IpLITR1.2a were aligned using the Clustal Omega software (http://www.ebi.ac.uk/Tools/msa/clustalo). (*) represent the same amino acid and (–) represents the absence of an amino acid from IpLITR1.2a that corresponds to IpLITR1.1b. The 'TM' overlined in black represents the transmembrane region of both protein sequences. 'itim?' represents tyrosine embedded in motifs that share similarities to canonical immunoreceptor tyrosine-based inhibitory motifs (ITIMs). 'ITSM' represents an immunoreceptor tyrosine-based switch motif.

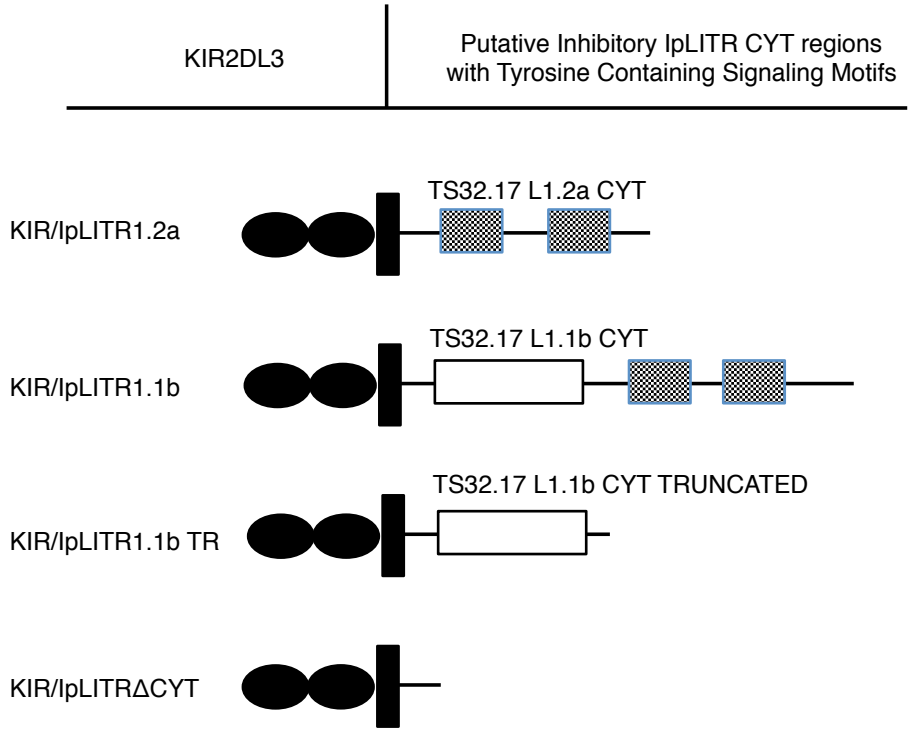

Figure 4.2. Schematic representation of KIR/IpLITR chimeric expression constructs. The extracellular region and transmembrane segment of human KIR2DL3 (NCBI accession number: U24074) were fused to the full-length cytoplasmic regions of the catfish LITR TS32.17 L1.1b, KIR/IpLITR1.1b (NCBI accession number: ABI16050) and TS32.17 L1.2a, KIR/IpLITR1.2a (NCBI accession number: ABI16051). A truncated CYT region of IpLITR TS32.17 L1.1b was also generated and designated as KIR/IpLITR1.1b TR. Tyrosine (Y) residues within each of the CYT region are shown and those present within ITIM-like motifs are indicated as shaded boxes. Tyrosine residues not within ITIM-like motifs are drawn as a white box. KIR/LITRΔCYT contains 28 amino acid residues within its CYT region and is devoid of tyrosines.

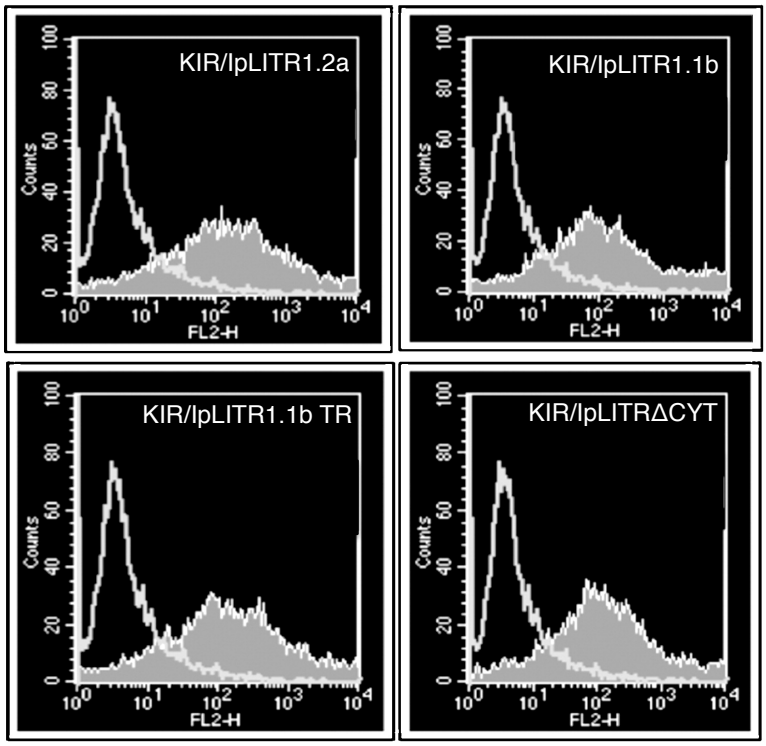

Figure 4.3. Cell surface expression of KIR/IpLITR constructs. A) Flow cytometric analysis of HEK 293T cells individually transfected with KIR/IpLITR 1.2a, 1.1b, 1.1b TR, or ΔCYT followed by staining with DX27 mAb and a goat anti-mouse IgG coupled to PE. Open histogram indicates mock-transfected cells and shaded histograms represent HEK 293T cells transiently transfected with each construct. Results are representative of three independent experiments.

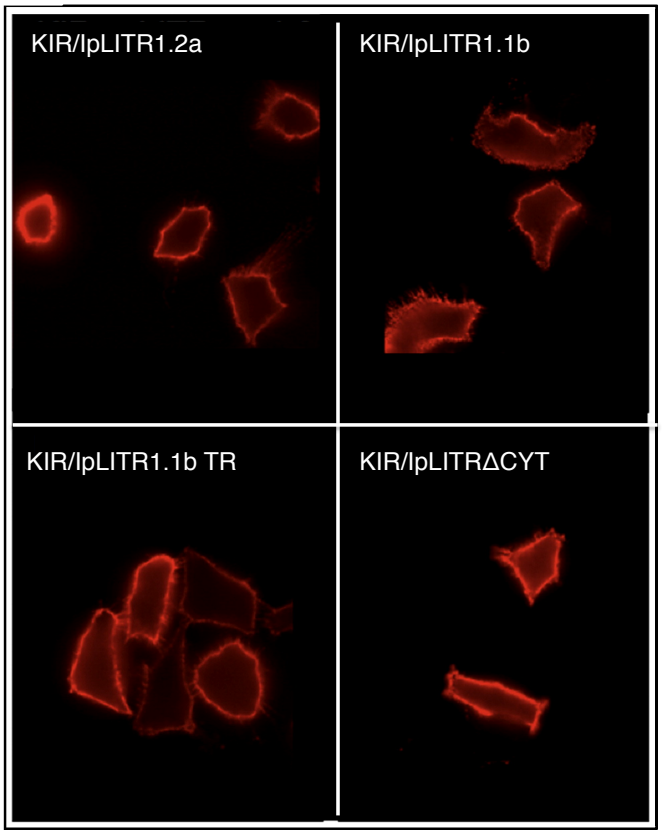

Figure 4.4. Fluorescent microscopy examination of cell surface expression of KIR/IpLITR constructs. HeLa cells individually transfected with KIR/IpLITR 1.2a, 1.1b, 1.1b TR, or ΔCYT followed by staining with DX27 mAb and a PE conjugated goat anti-mouse IgG. Results are representative of two independent experiments.

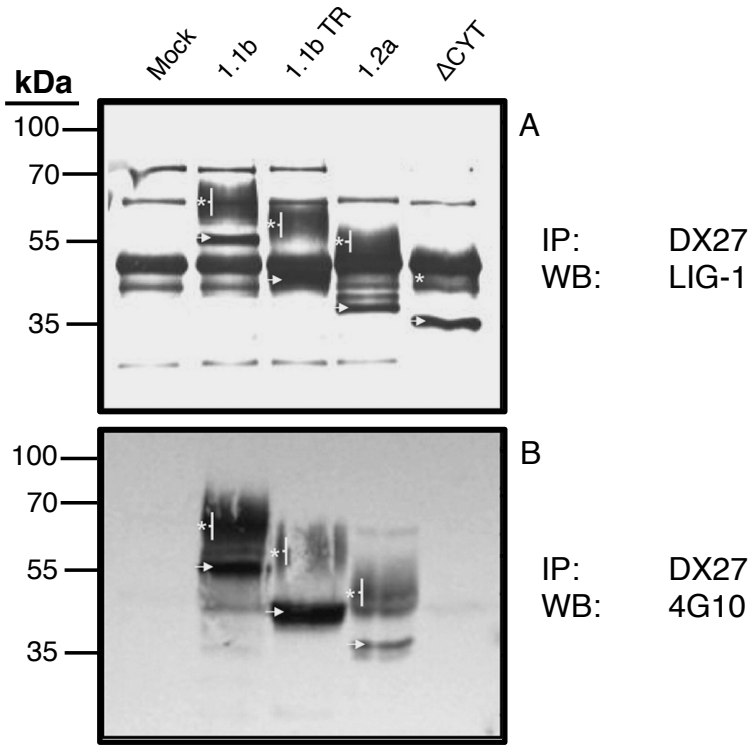

Figure 4.5. Immunoprecipitation of tyrosine-phosphorylated KIR/IpLITR constructs following pervanadate stimulation. HEK 293T cells were transiently transfected with KIR/IpLITR 1.2a, 1.1b, 1.1b TR, or ΔCYT and after 48 h the cells were treated for 15 min with pervanadate, lysed and then immunoprecipitated with DX27. Samples were separated on a 10% SDS-PAGE gel under reducing conditions, transferred to nitrocellulose and probed with either a biotin-conjugated anti-phosphotyrosine mAb, 4G10 (A) or with the anti-KIR mAb, LIG-1 (B). Two major phosphoprotein stained bands were observed for KIR/IpLITR 1.2a, 1.1b, 1.1b TR, which are indicated by an arrow (smaller band) and an asterisk (larger diffuse staining band) in (A). Mock-transfected cells and those transfected with KIR/IpLITRΔCYT did not demonstrate any phosphotyrosine staining after pervanadate treatment. A similar staining pattern for each construct was observed in (B) with the addition of some non-specific bands as indicated in the mock lane. Results are representative of two independent experiments.

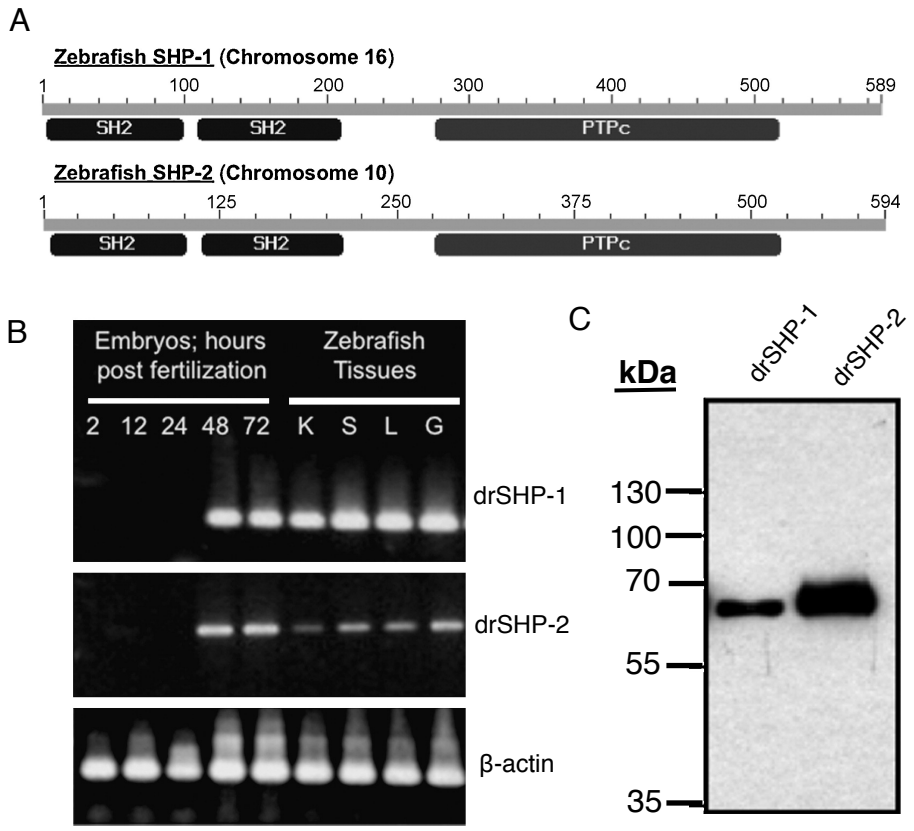

Figure 4.6. Characterization of zebrafish SHP-1 and SHP-2. Zebrafish SHP-1 and SHP-2 cDNAs were identified by BLAST searches using mammalian phosphatase protein sequences as queries. (A) Zebrafish SHP-1 and SHP-2 are 589 and 594 amino acid proteins, respectively, and both encode two N-terminal Src homology 2 (SH2) domains and a protein tyrosine phosphatase (PTP) catalytic domain. (B) RT-PCR analysis of SHP-1 and SHP-2 expression in developing zebrafish embryos (2–72 h post fertilization) and in adult tissues relative to the template control gene, β-actin. Tissues examined were kidney (K), spleen (S), liver (L), and gill (G). (C) Western blot detection of zebrafish SHP-1 and SHP-2 fusion protein constructs containing a C-terminal 3XFLAG epitope tag in the cellular lysates of HEK 293T transiently transfected with zebrafish SHP-1 and zebrafish SHP-2 and then immunoprecipitated with the anti-FLAG mAb M2. Blots were then probed with an anti-FLAG M2-HRP conjugated antibody.

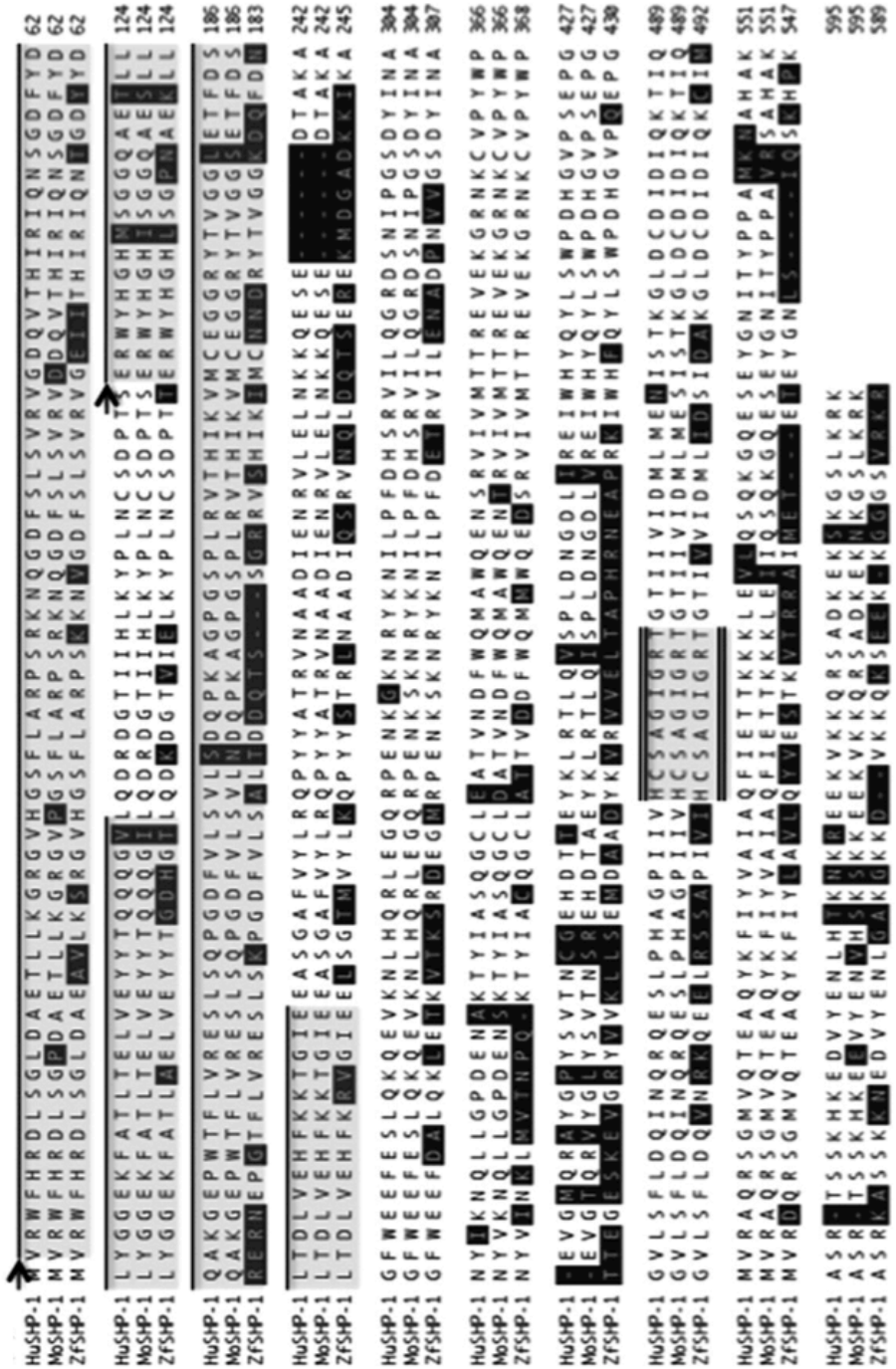

Figure 4.7. Amino acid alignments of human (Hu), mouse (Mo), and zebrafish (Zf) SHP-1. The dark grey shaded residues represent those that do not match the consensus sequence. The beginning of the N- and C-terminal SH2-domains is indicated with an arrowhead and the entire domains are shaded with light grey on the alignment. The PTP signature motif (HCSAGAGRT) is also indicated as a light shaded area surrounded by double lines.

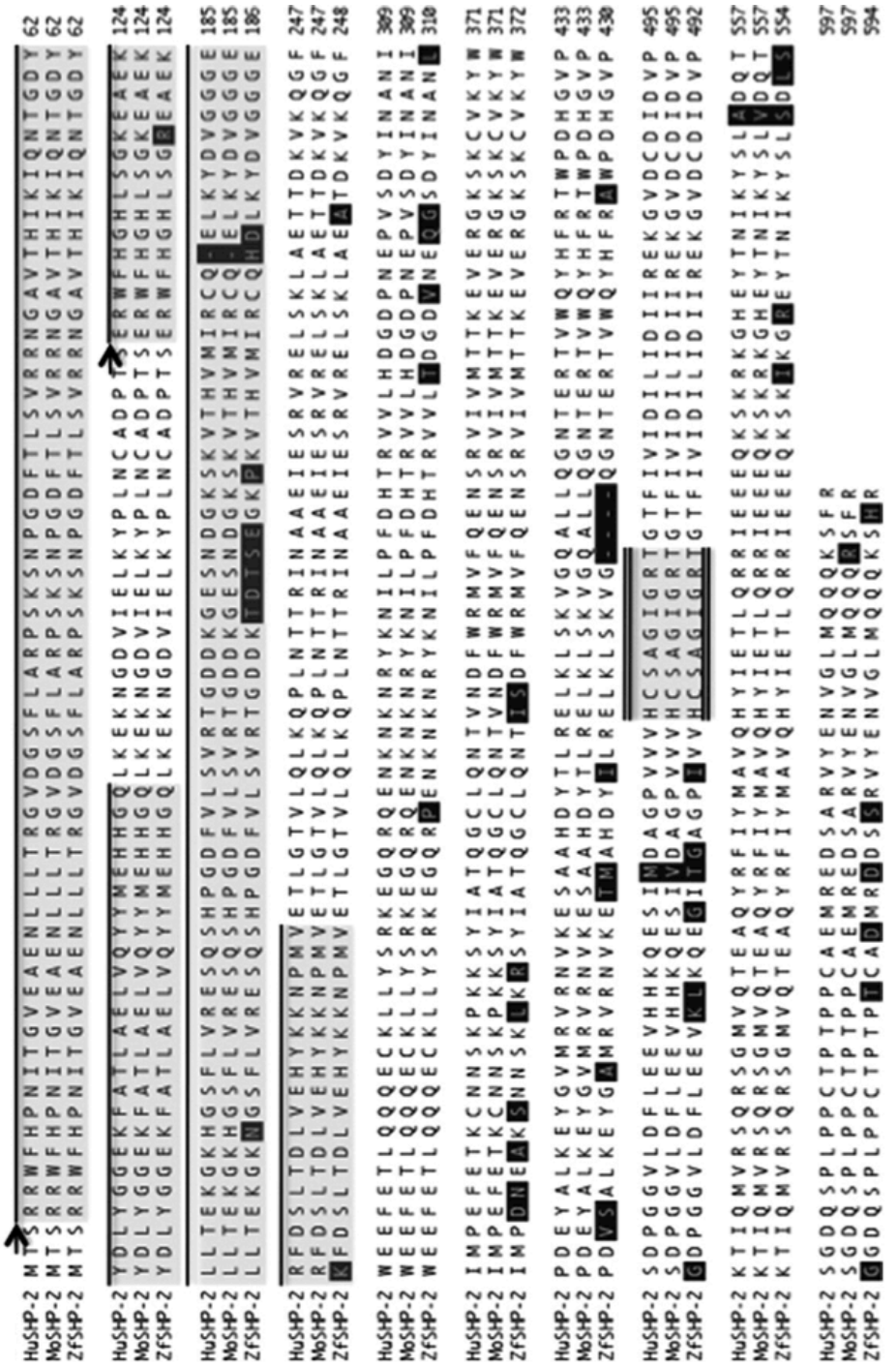

Figure 4.8. Amino acid alignments of human (Hu), mouse (Mo), and zebrafish (Zf) SHP-2. The dark grey shaded residues represent those that do not match the consensus sequence. The beginning of the N- and C-terminal SH2-domains is indicated with an arrowhead and the entire domains are shaded with light grey on the alignment. The PTP signature motif (HCSAGAGRT) is also indicated as a light shaded area surrounded by double lines.

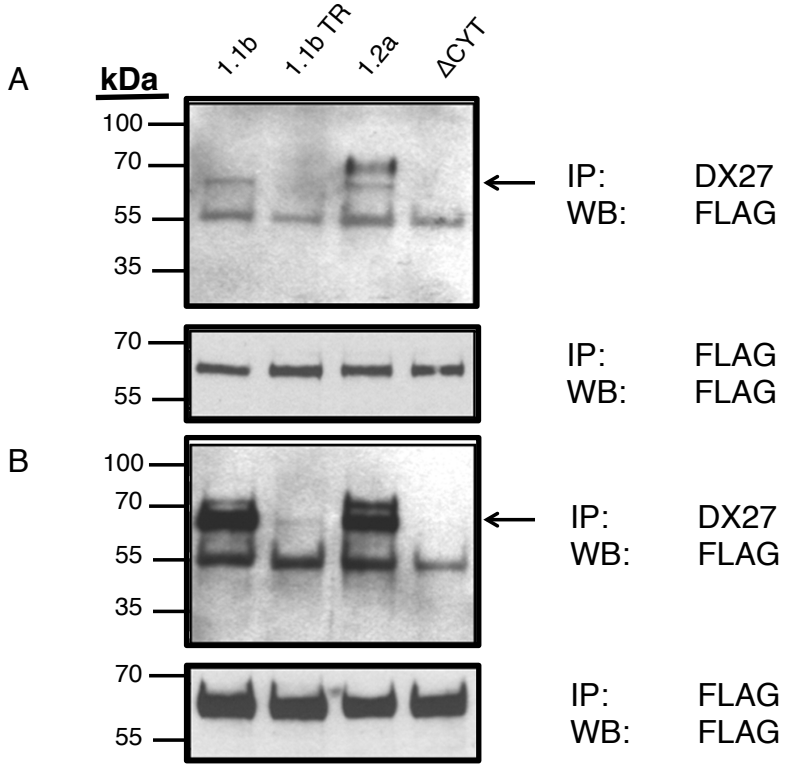

Figure 4.9. KIR/IpLITR 1.2a and 1.1b recruit zebrafish SHP-1 and SHP-2 following pervanadate-induced tyrosine phosphorylation. HEK 293T cells were transfected with KIR/IpLITR 1.2a, 1.1b, 1.1b TR, or ΔCYT and then co-transfected with the C-terminal 3XFLAG zebrafish SHP-1 fusion proteins. Forty-eight hours after transfection, the cells were pervanadate treated, lysed, and then immunoprecipitated with the anti-KIR mAb DX27 or anti-FLAG mAb. Samples were separated on a 10% SDS-PAGE gel under reducing conditions, transferred to nitrocellulose and probed with an anti-FLAG antibody conjugated to HRP for the detection of zebrafish SHP-1 (A) and SHP-2 (B) fusion proteins. The protein band corresponding to the predicted size of zebrafish SHP-1 (~65 kDa) is indicated with an arrow. Results are representative of three independent experiments performed.

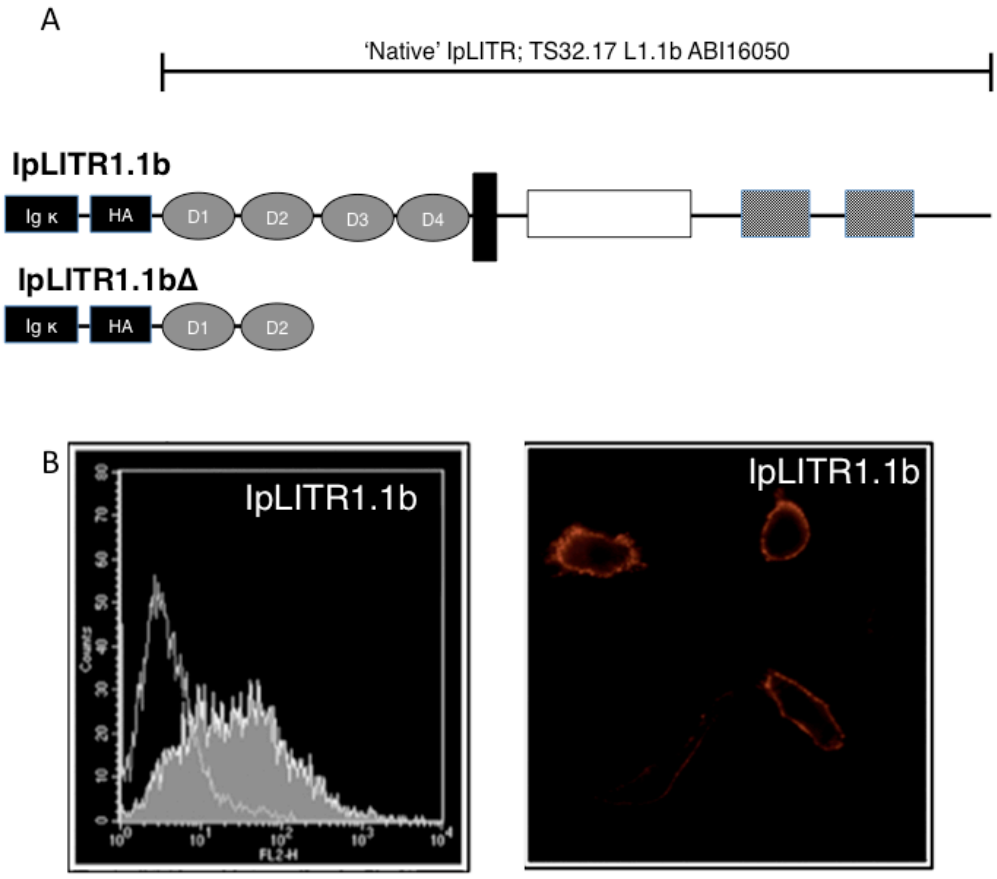

Figure 4.10. Native epitope-tagged IpLITR TS32.17 L1.1b is a surface-expressed protein that recruits SHP-1 and SHP-2 following tyrosine phosphorylation. (A) A schematic representation of the expression construct generated after cloning the TS32.17 L1.1b cDNA into the pDISPLAY expression vector. The full-length construct was designated as IpLITR1.1b and a truncated version of this construct due to incorporation of an in frame 'TGA' at amino acid position 216 was designated as IpLITR1.1bΔ. (B) Flow cytometry analysis of transfected HEK 293T cells followed by staining with an anti-HA mAb and a goat anti-mouse IgG coupled to PE (left panel). Open histogram indicates IpLITR1.1bΔ-transfected cells and shaded histogram represents HEK 293T cells transiently transfected with IpLITR1.1b. Note: For IpLITR1.1bΔ, mock-transfected, and isotype-stained cells M1 was <10%. Microscope analysis of HeLa cells transfected with IpLITR1.1b followed by staining with anti-HA mAb and a goat anti-mouse IgG coupled to PE.

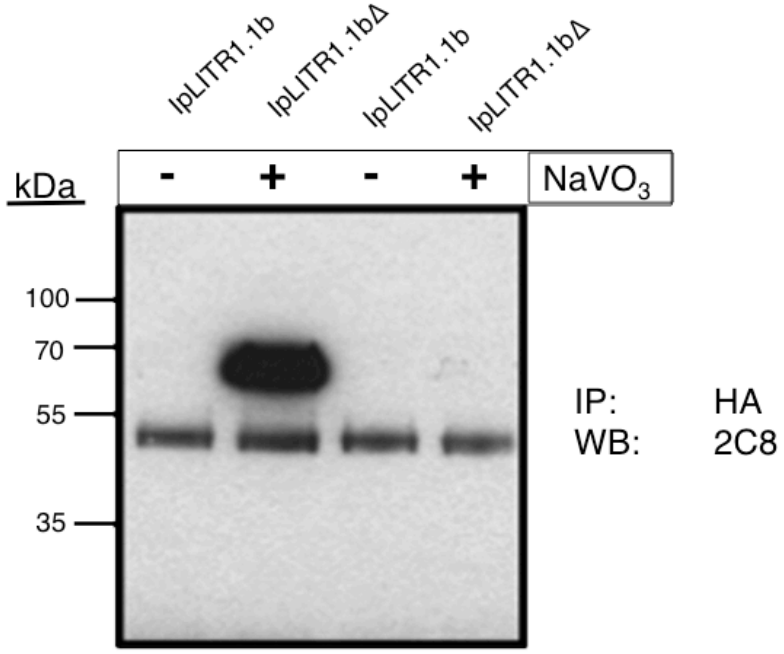

Figure 4.11. Native epitope-tagged IpLITR TS32.17 L1.1b is phosphorylated at tyrosine residues following pervanadate stimulation. TS32.17 L1.1b IpLITR1.1b and IpLITR1.1bΔ were transfected into HEK 293T cells. Forty-eight hrs after transfection the cells were pervanadate treated (+) or incubated with D-PBS (-), lysed, and then immunoprecipitated with an anti-HA mAb. Samples were separated on a 10% SDS-PAGE gel under reducing conditions, transferred to nitrocellulose and blots were probed with either an anti-phosphotyrosine mAb to detect tyrosine-phosphorylated TS32.17 L1.1b. Results are representative of three independent experiments performed.

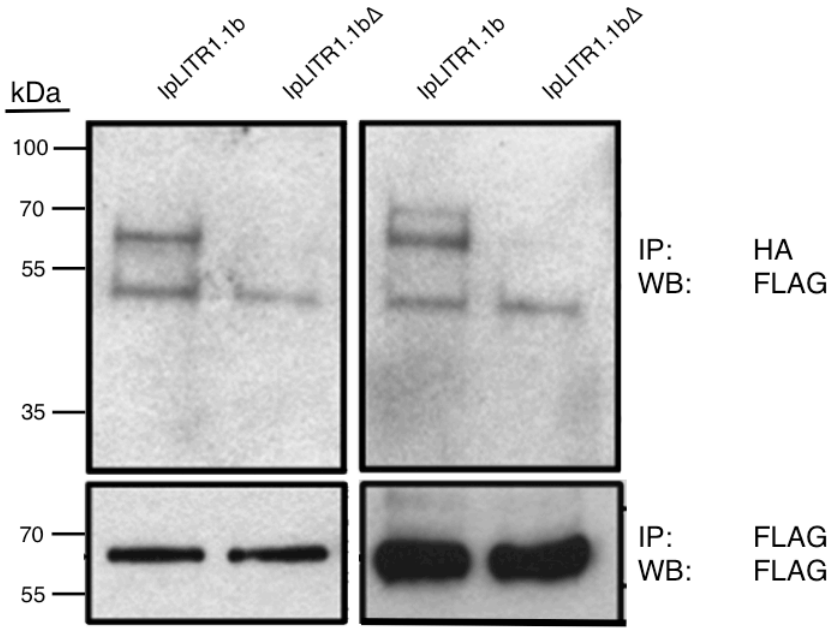

Figure 4.12. Native epitope-tagged IpLITR TS32.17 L1.1b recruits SHP-1 and SHP-2. TS32.17 IpLITR1.1b and IpLITR1.1bΔ were co-transfected into HEK 293T cells with the C-terminal 3XFLAG zebrafish SHP-1 or SHP-2 fusion proteins. Forty-eight hrs after transfection the cells were pervanadate treated, lysed, and then immunoprecipitated with either anti-HA mAb or anti-FLAG mAb. Samples were separated on a 10% SDS-PAGE gel under reducing conditions, transferred to nitrocellulose and blots were probed with an anti-FLAG antibody conjugated to HRP for the detection of zebrafish SHP-1 (left blot) or zebrafish SHP-2 (right blot) fusion proteins. Results are representative of two independent experiments performed.

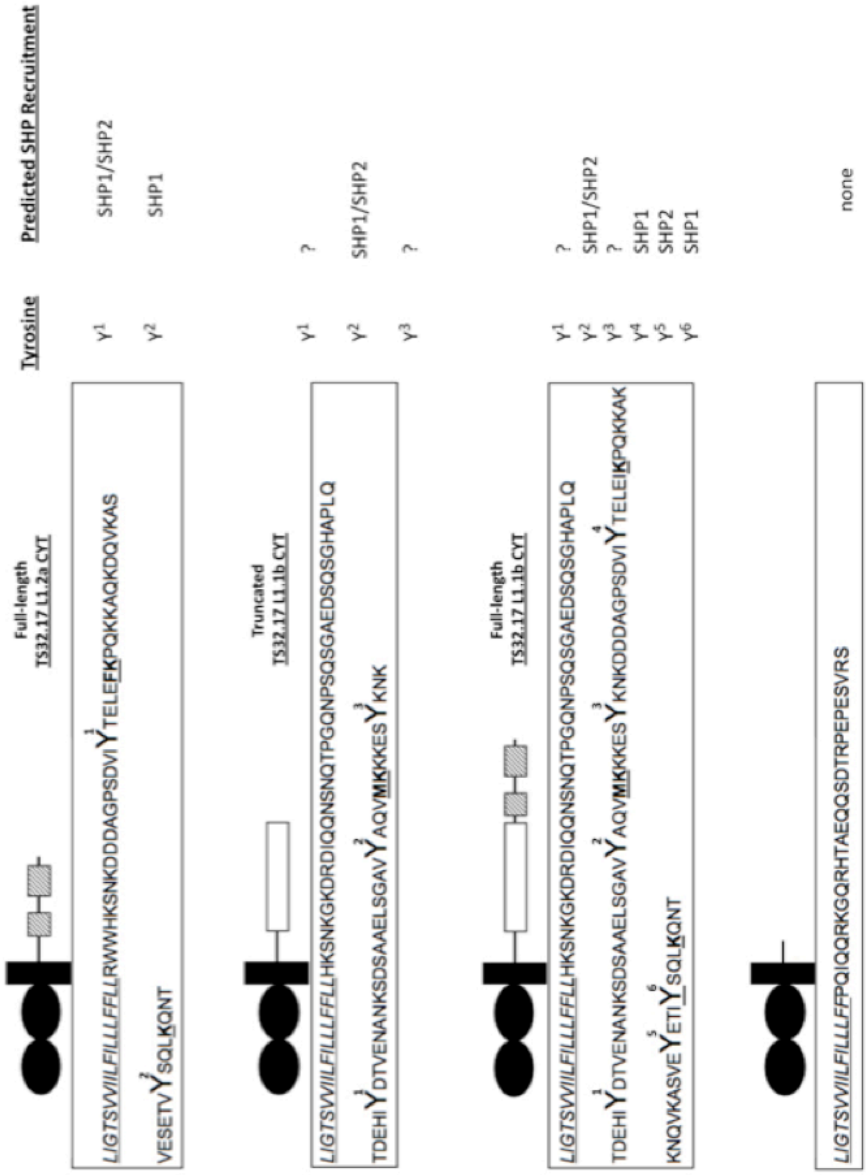

Figure 4.13. SHP-1 and SHP-2 binding potential of tyrosine-containing IpLITR CYT regions. For each construct, the CYT tyrosine residues were examined for their SHP-1 and SHP-2 recruitment potential according to the presence of specific amino acid residues at the Y −2 to +6 positions. The TM segment of the KIR2DL3 for each construct is underlined and in italic and the full amino acid sequence of each IpLITR CYT region is shown. Tyrosines are numbered based on their proximity to the TM segment (i.e. Y^1 is the membrane proximal residue) and key residues that facilitate SHP-1 and/or SHP-2 binding at the Y +4 to +6 positions are underlined and in bold. Indicated on the right are the predicted SHP-1 and/or SHP-2 recruitment potential of each tyrosine residue in all constructs examined.

CHAPTER V

IpLITR1.1b AND IpLITR1.2a INHIBIT CYTOTOXICITY IN A SHP-1-DEPENDENT AND –INDEPENDENT FASHION

Portions of this chapter have been published:
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5.1. Introduction

Inhibitory receptors establish the activation threshold of immune cells and attenuate stimulatory receptor-induced effector functions. The inhibitory capacity of these receptors is primarily dependent on the presence of ITIMs within their CYTs [50-52, 277]. Ligand-induced phosphorylation of the tyrosine residue embedded within ITIMs (S/I/V/LxYxxI/V/L; where x indicates any amino acid) leads to the recruitment of SH2 domain-containing cytoplasmic phosphatases (SHP-1, SHP-2, SHIP), which dephosphorylate various intracellular activation signaling intermediates [51, 266]. In chapter IV, I demonstrated that the CYT of IpLITR1.2a and IpLITR1.1b recruit zebrafish SHP-1 and SHP-2 [278]. This same recruitment was not observed with the truncated KIR/IpLITR1.1b TR, which contains three tyrosine residues but no canonical ITIMs [278]. Despite this biochemical information, it remains unknown whether or not the CYT regions of IpLITR1.1b and IpLITR1.2a have the ability to functionally inhibit immune cell effector responses.

To date, there is only one published account of functional inhibition by teleost immunoregulatory receptors. Yoder *et al*. published a report wherein they expressed the ITIM-bearing receptor, NITR3.1, on the surface of NK92 cells and through Ab cross-linking (in the presence of the Raji tumor cell line) they demonstrated a decrease in the phosphorylation of MAPK [147]. The phosphorylation of MAPK is a well-characterized signaling event in the effector responses of NK cells [44]. In addition to this work in teleost, Yu *et al*. revealed that a the CYT region of a sea lamprey TCR-like molecule containing two ITIMs, was capable of inhibiting BCR-induced calcium mobilization and Erk/Akt/JNK activation in a mouse B cell line [279]. In the CHIR family of IgSF receptors, it was discovered that, following Ab crosslinking, CHIRB2 recruits SHP-1 and SHP-2, which leads to a decrease in proliferation as well as reduced calcium mobilization in stably transfected DT40 cells, a clonal chicken B-cell line [128]. These are all examples that reinforce the idea that ITIM mediated inhibition by IgSF immunoregulatory receptors is a conserved strategy in vertebrates.

Expanding on my findings from chapter IV, the focus of this chapter was to examine the functional inhibitory capabilities of IpLITR1.1b and IpLITR1.2a by addressing the following questions: (i) do the ITIM-bearing CYT regions of IpLITR1.2a and IpLITR1.1b inhibit cellular immune responses; (ii) what are the inhibitory signaling pathways used by IpLITR1.2a and 1.1b; and (iii) is there an inhibitory function mediated by the unique tyrosine-containing, membrane-proximal CYT region of IpLITR1.1b? My hypothesis was IpLITR1.1b and 1.2a are functional inhibitory receptors and this inhibitory ability requires the ITIMs in the CYTs. This hypothesis is supported by the findings from the previous chapter, where I demonstrated that the CYT of IpLITR1.1b

and 1.2a are capable of recruiting SHP-1 and SHP-2. Herein I show that these receptors do indeed function as potent inhibitors of immunoregulatory signaling as they efficiently shut down lymphocyte-mediated cellular cytotoxicity (i.e. target cell killing) upon ligand engagement.

To investigate my hypothesis, I began by testing transfection methods to successfully express the KIR/IpLITR chimeras on the surface of a clonal mammalian NK cell line in order to examine the function of these ITIM-bearing IpLITRs. In the end, infection of primary mouse NK cells with a vaccinia virus (VV) encoding the KIR/IpLITR chimeras was a successful strategy for surface expression of the receptors and ability to measure killing responses using the ^{51}Cr release assays. With this expression system optimized and coupled with a reliable cytotoxicity assay, examination of the functional abilities of IpLITR1.1b and IpLITR1.2a CYT regions was possible. This chapter describes the results of experiments designed to examine the inhibitory activity of IpLITR1.1b and IpLITR1.2a on the killing response of mammalian NK cells. I found that the CYT regions of IpLITR1.2a and IpLITR1.1b do indeed inhibit NK cell killing responses at comparable levels with the prototypical SHP-1 recruiting inhibitory receptor, KIR2DL3. The introduction of an rVV encoding a DN-SHP-1 revealed that the inhibitory activity of IpLITR1.2a was SHP-1-dependent while IpLITR1.1b and IpLITR1.1b TR could inhibit killing in a SHP-1-independent manner. Coupled with the results from Chapter IV wherein the TM proximal CYT region does not recruit SHP-1 or SHP-2, the results in this chapter support that the TM proximal CYT region of IpLITR1.1b also initiates inhibitory signaling through a mechanism that does not require SHP-1 recruitment.

5.2. Results

5.2.1. Examination of IpLITR-mediated inhibition of NK cell killing responses using YTS cells.

The first step towards demonstrating a functional inhibitory response mediated by the KIR/IpLITR chimeric constructs was to express these receptors in a functional immune cell line and then measure any inhibitory effects mediated through the CYT region as initiated by ligand recognition of the extracellular KIR2DL3 region. YTS cells were transduced and then bulk-sorted on a FACSaria. Surface expression was observed on bulk-sorted YTS cells transduced with KIR2DL3, KIR/IpLITR1.1b, KIR/IpLITR1.1b TR, and KIR/IpLITRΔCYT as demonstrated by staining transduced YTS cells with DX27 and measuring fluorescence with flow cytometry (Figure 5.1). Although YTS cells transduced with KIR/IpLITR1.2a survived through selection, the chimeric receptor was not detected on the cell surface following transduction (data not shown). Cytotoxicity assays were then performed by incubating the transduced YTS cells with the MHC-I deficient target cell line 721.221 and 721.221 expressing the cognate KIR2DL3 ligand, HLA-Cw3 (.221-Cw3) using a standard ^{51}Cr-release assay [280] (Figure 5.2). Cytolysis assays were conducted with the transfected YTS cells and (Figure 5.2). Non-transfected YTS cells killed 721.221 cells and .221-Cw3 cells at similar levels at all effector-to-target (E:T) ratios tested (1:1, 3:1, and 10:1) (Figure 5.2). As expected, YTS cells expressing KIR2DL3 demonstrated efficient killing of the target cell line 721.221 while cytotoxicity was inhibited when the cells encountered .221-Cw3 (Figure 5.2). YTS cells transduced with KIR/IpLITR1.1b did not demonstrate efficient killing of 721.221

cells or .221-Cw3 as neither target was killed at levels greater than 10% at the highest E:T of 10:1 (Figure 5.2). However, IpLITR1.1b TR, which contained no classical ITIMs, demonstrated efficient killing of 721.221 target cells (~30% at the ratio of 10:1) and a diminished cytotoxicity with .221-Cw3 target cells (~11% at 10:1) (Figure 5.2). This inhibition of cytotoxicity was similar to the inhibition demonstrated by the wild-type KIR2DL3 upon engagement with HLA-Cw3,with specific killing as 6% at an E:T of 10:1, while killing of 721.221 cells was found to be ~35% (Fig. 5.2). To further confirm that YTS cells expressing KIR/IpLITR1.1b to were incapable of killing target cells,multiple subclones were generated and tested for their ability to kill 721.221 cells. Before the cytotoxicity assay was performed, subclones were measured for matched surface expression of chimeric receptors (Fig. 5.3). Figure 5.4 shows that a representative sample of subcloned YTS expressing KIR/IpLITR1.1b all demonstrated negligible killing ability with levels of 5%, 4%, 10%, 5%, and 3% for clones D, E, F, G, and H, respectively, while KIR/IpLITR1.1b TR, KIR/IpLITRΔCYT, and nontransduced YTS cells all killed target cells above 27%.

5.2.2. Examination of IpLITR-mediated inhibition of cytoxicity responses using NK92 cells.

The human NK-like cell line, NK92, has been shown previously to be an effective tool in demonstrating the function of inhibitory receptors as was the case when these cells were used to demonstrate the recognition of KIR2DL4 for the non-classical MHC-I, HLA-G through infection with recombinant vaccinia virus (rVV) encoding KIR2DL4 [102]. The wildtype KIR2DL3, KIR/IpLITR1.1b, and KIR/IpLITRΔCYT were subcloned into the plasmid, pSC66, and used to generate rVV. Purified viruses encoding the empty vector pSC66, KIR2DL3, KIR/IpLITR1.1b, or KIR/IpLITR1.1bΔCYT were

then used to infect NK92 cells. Following infection, NK92 cells were monitored for surface expression of receptors by staining with DX27 and measuring with flow cytometry. I observed that surface expression was comparable for all constructs expressed on NK92 cells (Fig. 5.5). Infected NK92 cells were then combined with the same target cell lines as described above to measure killing responses. There was no significant difference in the killing response of NK92 cells infected with rVV encoding the empty vector pSC66 (Fig. 5.6). As expected, KIR2DL3 provided strong inhibition of .221-Cw3 killing but not 721.221 killing (Fig. 5.6). Unlike what I observed for YTS cell killing, NK92 expressing KIR/IpLITR1.1b inhibited the killing of target cells at a level comparable to KIR2DL3. In addition, there was also a ~20% drop in specific lysis with NK92 expressing KIR/IpLITRΔCYT (Fig. 5.6), which was completely unexpected. This result was surprising because KIR/IpLITRΔCYT contains a very short CYT region that is devoid of tyrosine residues and my prediction was that, consistent with the inhibition of cytotoxicity in YTS cells and the inability to recruit SHP-1 or SHP-2 (Chapter IV), this construct would play the role of a negative control to confirm that all inhibition was mediated through signals originating in the CYT region. This inhibition may be due to the engagement of a co-receptor and will be further elaborated in the Discussion section.

5.2.3. Blocking with the pan-reactive MHC-I Ab W6/32 differentiates inhibition by KIR/IpLITRΔCYT compared to KIR2DL3 and KIR/IpLITR1.1b

To ensure that the reduction in lysis by KIR/IpLITR1.1bΔCYT was due to a specific interaction between the KIR2DL3 extracellular region and HLA-Cw3 expressed on the target cells, I performed the same ^{51}Cr release assays in the presence of the pan-reactive HLA mAb W6/32, which has been shown to bind the α3 and β2M region of

MHC-I [281]. This antibody binds to MHC-I molecules at a site that is separate from where KIR engage MHC-I so reversion of cytotoxicity in the presence of this antibody would suggest that other surface-expressed molecules may be involved in this response [282]. The isotype of W6/32 is IgG2A and so a non-specific IgG2A antibody was used as an isotype control. I observed that there was not a significant change in the level of cytotoxicity for NK92 cells expressing KIR2DL3 and KIR/IpLITR1.1b in the presence of W6/32 (Fig. 5.7). Conversely, NK92 cells expressing KIR/IpLITRΔCYT demonstrated a significant reversion of cytotoxicity from 14% to 32% in the presence of W6/32 but no change in cytotoxicity in the presence of the isotype control (Fig. 5.7).

5.2.4. Examination of IpLITR-mediated inhibition of cytotoxicity using primary mouse NK cells.

Assays using the NK92 cell line were halted due to the possibility of interference from co-receptors. To avoid the interfering components from NK92 cells, which were possiby other human surface receptors, I substituted the NK92 cells for splenic-derived primary mouse NK cells. These cells are potent mediators of cytotoxicity and the chimeric KIR/IpLITR receptors could be transduced via infecting the cells with rVV encoding the various expression chimeras similar to the NK92 cell line [93]. Mouse NK cells were infected with rVV encoding an empty pSC66 vector, the pSC66 vector encoding KIR2DL3, or the pSC66 vectors encoding the KIR/IpLITR1.2a, KIR/IpLITR1.1b, or KIR/IpLITRΔCYT. Following infections with rVV encoding the various constructs, NK cells were monitored for surface receptor expression by flow cytometry using the DX27 mAb. All receptors were detected at comparable levels on the surface of NK cells, whereas no expression was observed in the empty vector control,

pSC66 (Fig. 5.8). KIR/IpLITR1.1b demonstrates slightly reduced expression levels when compared to the other constructs. However, as described below, this did not influence its inhibitory capabilities.

Cytotoxicity assays were then performed by incubating the rVV-infected NK cells with 721.221 and .221-Cw3 targets using the ^{51}Cr-release assay. Prior to mixing, the target cells were coated with an anti-MHC-II antibody in order for the mouse NK cells to engage the targets through the stimulatory IgG receptor CD16. For the NK cells infected with rVV containing the empty pSC66 vector, I observed that 721.221 and .221-Cw3 target cell killing was most effective (~50%) at the highest E:T ratio tested (Fig. 5.9). I then tested the cytotoxic response of pSC66-KIR2DL3 infected NK cells and observed increased killing of 721.221 targets at the increasing E:T ratios with ~48% specific killing observed at the ratio 10:1 (Fig. 5.9). Combination of KIR2DL3 with the .221-Cw3 cells resulted in % killing that was significantly reduced at 11%, consistent with what I demonstrated before in YTS cells and NK92 cells (Fig. 5.9). As observed with NK cells expressing KIR2DL3, both KIR/IpLITR 1.2a and KIR/IpLITR1.1b demonstrated a significant inhibition of killing responses when they engaged 221.Cw3 (~10-12%) in comparison to 721.221, whereas NK cells expressing KIR/IpLITRΔCYT demonstrated no difference between the % killing of 721.221 cells and .221-Cw3 target cells (Fig. 5.9).

5.2.5 The membrane proximal cytoplasmic region of IpLITR1.1b inhibits NK cell-mediated cellular cytotoxicity

As previously reported in Chapter IV, the CYT region of IpLITR1.1b has a unique TM proximal region, which contains two tyrosine residues (Y^{433} and Y^{453}) in the context of a sequence of residues that do not fit within the classical definition of an ITIM.

I also demonstrated in Chapter IV that this CYT region recruits relatively little to no teleost SHP-1 or SHP-2 in comparison to the SHP-recruiting ability of its TM distal ITIMs in IP experiments. When KIR/IpLITR1.1b TR was expressed in YTS cells it inhibited the killing of .221-Cw3 target cells (Fig. 5.2). With this in mind, I wanted to confirm whether KIR/IpLITR1.1b TR would have a strong inhibitory potential in primary mouse NK cells. Surface expression of KIR/IpLITR1.1b TR was similar to that of KIR2DL3 and KIR/IpLITR1.1b as measured by flow cytometry (Fig. 5.10) NK cells expressing KIR/IpLITR1.1b TR mediated the inhibition of lysis (Fig. 5.11). These levels of inhibition were similar to those observed using KIR/IpLITR1.1b and the wild-type KIR2DL3 (Figure 5.11).

5.2.6 Inhibition of cytotoxicity is due to specific interactions between KIR/IpLITR chimeras with the ligand HLA-Cw3

As shown in Figure 5.12, the inhibitory functions of KIR2DL3, KIR/IpLITR1.2a, KIR/ IpLITR1.1b, and KIR/IpLITR1.1b TR, were all due to specific interactions with HLA-Cw3. Specifically, by performing cytotoxicity assays in the presence of the DX27 mAb, which binds to a specific site on KIR2DL3 to interfere with its engagement of MHC-I, the % killing of 221.Cw3 target cells (Fig. 5.12B; grey bars) was reverted back to a %killing more similar to 721.221 target cells(Fig.. 5.12A; black bars). However, no reversion of target cell killing by the NK cells was observed in the presence of the IgG2a isotype control antibody (Fig. 5.12B).

5.2.7. The chemical SHP-1 inhibitors, sodium stibogluconate and PTP I inhibitor, are not effective in cytotoxicity assays

In order to further examine IpLITR-mediated inhibitory signaling and its dependence on intracellular phosphatases, I attempted to block SHP-1 function using chemical inhibitors and the use of a DN-SHP-1. As shown in Figure 5.13, there was no difference in the inhibitory potential of the IpLITR chimeras in the presence of 11 μM SSG at an E:T ratio of 10:1. Killing of 721.221 cells was established at levels similar to the previous cytotoxicity assays and inhibition of .221-Cw3 target cell killing by KIR2DL3, KIR/IpLITR1.2a, KIR/IpLITR1.1b, and KIR/IpLITR1.1b TR was also reproduced (Fig. 5.13). I also performed the NK cytotoxicity assay with NK cells expressing the human inhibitory receptor LILRB1, an IgSF inhibitory receptor demonstrated to recruit SHP-1 [114], and found that inhibition by this receptor was also not affected by SSG treatment. (Fig. 5.14 and 5.15).

To determine if SSG was effectively inhibiting SHP-1 function, I also tested YTS cells transduced with a chimeric receptor that consisted of the EC and TM regions of KIR2DL1 with the CYT region being replaced by full-length SHP-1 (WT) or the capture mutant (DA) to serve as a negative control. Figure 5.16A presents a schematic diagram of the two different chimeric receptors that were transduced into YTS cells. I then measured surface expression of both YTS-KIR2DL1/SHP-1 WT and YTS-KIR2DL1/SHP-1 DA by flow cytometry and staining with an anti-KIR2DL1 mAb, EB6 (Fig. 5.16B). Both transduced YTS cells demonstrated similar levels of chimeric

receptor expression on the cell surface. These YTS cells were then tested for their cytotoxic responses to the target cells 721.221 and .221-Cw15, (HLA-Cw15 being one of the HLA ligands for KIR2DL1). YTS-KIR2DL1/SHP-1 (WT) demonstrated efficient killing of 721.221 cells while inhibited killing of .221-Cw15 cells (Fig. 5.16C, top panel). YTS-KIR2DL1/SHP-1 (DA) did not demonstrate any difference between % killing whether the target cell line was 721.221 or .221-Cw15 (Fig. 5.16C, bottom panel). With these results, I confirmed that YTS cells transduced with the chimeric KIR2DL1/SHP-1 (WT) and (DA) were both functional cytotoxic cells and the functional inhibition was dependent on SHP-1-mediated signaling.

I then tested whether SSG treatment could specifically inhibit SHP-1 function in cytotoxicity assays. I found that SSG had no effect on the cytotoxic capacity of transduced YTS cells vs. both 721.221 cells and .221-Cw15 cells (Fig. 5.17). I tested multiple concentrations of SSG, from 2μM to 2000μM, and found no difference between any of these concentrations. With the cells seeming unresponsive to SSG, I also employed another SHP-1 inhibitor, PTP 1. As shown in Figure 5.18, I found that treatment with low concentrations of PTP I inhibitor led to a loss of cytotoxicity by effector cells. As the concentration increased, the amount of specific killing increased at a level consistent between the two different transduced YTS cell lines at ~ 60% (Fig. 5.18). At first glance this would seem to suggest that at high levels of treatment, PTP I inhibitor can block SHP-1 recruitment and signaling leading to a reversion of the inhibitory signaling. Further examination of the viability of the effector and target cells in the cytotoxicity assay resulted in a realization that high concentrations of PTP I inhibitor led to cytotoxicity of both target and effector cells as discovered by trypan

staining following the four-hour incubation of targets and effectors (data not shown). Further confirmation of the toxicity of PTP I inhibitor came from my attempts to use it with rVV infected NK cells expressing the KIR2DL3, KIR/IpLITR1.2a, KIR/IpLITR1.1b, KIR/IpLITR1.1b TR, KIR/IpLITRΔCYT and found that significant toxicity occurred with PTP I treatments although there was no change in viability when cells were solely treated with DMSO (Figure 5.19).

5.2.8 DN-SHP-1 abrogates the inhibitory effects of IpLITR1.2a, but not IpLITR1.1b, in mouse NK cells

Co-infection of NK cells with an rVV encoding the empty vector pSC66, pSC66-KIR2DL3, or pSC66-KIR/IpLITR chimeras in combination with a rVV encoding a DN-SHP-1 recombinant human protein was then performed as an alternative to the chemical blockers of SHP-1. The DN-SHP-1 used in these experiments encode two N-terminal SH2 domains, the protein tyrosine phosphatase domain (PTP), and the C453 to S453 mutation that renders this protein catalytically inactive (Fig. 5.20A;[50]). Also shown is a WB demonstrating that in NK cells, recombinant DN-SHP-1 can be identified as an ~ 70 kDa protein, which is slightly larger than the endogenous SHP-1 observed in the sample lane consisting of lysate from NK cells infected with an rVV encoding the empty vector pSC66 (Fig. 5.20B).

To normalize the level of infection between experimental groups (i.e. to ensure that each cell was infected with similar amounts of rVV), I co-infected NK cells with the pSC66 empty vector in combination with pSC66-KIR2DL3, pSC66-KIR/IpLITR1.2a, pSC66-KIR/IpLITR1.1b, or pSC66-KIR/IpLITR1.1b TR constructs. When examined by flow cytometry using DX27 mAb, the NK cells that were co-infected with empty pSC66 or DN-SHP-1 in conjunction with the various KIR/IpLITR chimeras demonstrated

similar levels of receptor expression on the cell surface (Fig. 5.20C). Then using the ^{51}Cr release assay to assess NK cell cytotoxicity, I observed that the catalytically inactive DN-SHP-1 significantly reversed the inhibition of 721.221 killing mediated by KIR2DL3- and KIR/IpLITR1.2a- expressing NK cells when incubated with .221-Cw3 cells (Fig. 5.21). For example, NK cells expressing KIR2DL3 that were co-infected with empty pSC66 killed 30% of 721.221 cells and only 10% of targets if they expressed HLA-Cw3. Conversely, when co-infected with DN-SHP-1, the KIR2DL3 expressing NK cells killed 43% of 721.221 targets and 37% of targets if they expressed HLA-Cw3. This reverted killing of 721.221-HLA-Cw3 targets by KIR2DL3-expressing NK cells co-infected with empty pSC66 (10%) vs. DN-SHP-1 (37%) is statistically significant ($p < 0.05$; Fig. 5.21). A DN-SHP-1-dependent reversion of inhibitory receptor function was not exclusive to KIR2DL3. When I tested the KIR/IpLITR chimeras, I observed that NK cells expressing KIR/IpLITR1.2a, and co-infected with rVV encoding pSC66 to normalize infection levels, killed 32.5% of 721.221 cells and only 9% of targets if they expressed HLA-Cw3. However, when co-infected with an rVV encoding DN-SHP-1, the KIR/IpLITR1.2a-expressing NK cells killed almost 50% of 721.221 targets and almost 40% of .221-Cw3 targets. Once again, this reverted killing of 721.221-HLA-Cw3 targets by KIR/IpLITR1.2a-expressing NK cells co-infected with pSC66 vs. DN-SHP-1 is statistically significant ($p < 0.05$; Fig. 5.21). Notably, neither KIR/IpLITR1.1b nor KIR/ IpLITR1.1b TR demonstrated a reversion of their inhibitory responses when the catalytically inactive DN-SHP-1 was present. This was evident by the absence of reversion of cytotoxicity when DN-SHP-1 was introduced to revert the killing of .221-Cw3 targets by KIR/ IpLITR1.1b- or KIR/IpLITR1.1b TR-expressing NK cells co-

infected with empty pSC66 vs. DN-SHP-1 (Fig. 5.21). However, KIR/IpLITR1.1b and KIR/IpLITR1.1b TR expressing NK cells still effectively killed MHC I-deficient targets.

5.4 Discussion

The data presented in this chapter demonstrates that IpLITR1.1b and 1.2a are functional inhibitory receptors capable of efficiently regulating cytotoxicity in mammalian NK cells. The first cell line that I chose to test for this set of experiments was the YTS cell line [283]. The YTS cell line is a transformed human NK-like cell line that does not express other inhibitory IgSF family members (including KIRs or LILRs) on the surface, making it an ideal candidate cell line to test a chimeric receptor that utilizes a KIR EC region. If the YTS line did endogenously express KIR2DL3 on the surface then it would be extremely difficult to discern the effects of signaling between the endogenous KIRs and the transduced chimeric KIR/IpLITRs. As it turned out, it proved impossible to engage KIR/IpLITR1.1b transduced YTS cells to demonstrate any significant amount of cytotoxicity to target cells. I do not have an answer to why this occurred but I can speculate that because the YTS cell line lacks many stimulatory and inhibitory immunoregulatory receptors and can engage targets solely through B7-CD28 engagement, this may not be a strong enough signal to activate the cell when KIR/IpLITR1.1b is expressed. KIR/IpLITR1.1b contains many recruitment motifs within its CYT region including two canonical ITIMs, an ITSM, and a region of ITIM-like and other unknown possible recruitment regions. This CYT region may be constitutively active and the signal to overcome its inhibitory potential must be more potent than what was delivered by the B7:CD28 engagement. An example of this constitutive inhibitory potential is demonstrated by Paired Ig-like receptor (PIR)-B. It has been shown that PIR-

B is constitutively active on macrophages and B-cells irrespective of the cell activation status [284].

The next cell line that was tested in order to demonstrate the inhibitory potential of the KIR/IpLITR constructs was the NK92 cell line. This is another transformed clonal human NK-like cell line that expresses an array of stimulatory receptors on the surface but it does not express any KIR members making it another suitable cell line to use to explore my objectives [285]. Surprisingly, when IpLITRΔCYT was transfected into NK92 cells it demonstrated inhibition of cytotoxicity when combined with .221-Cw3. This inhibition by KIR/IpLITR1.1bΔCYT was reverted when the cytotoxicity assay was repeated in the presence of a pan-reactive MHC-I mAb (W6/32). These results suggest that the mechanism of signaling by KIR/IpLITRΔCYT differs from that of the full-length KIR2DL3 and KIR/IpLITR1.1b, which were not affected by the addition of W6/32. A possible mechanism for this CYT-less inhibitory response has been proposed in a report by Kirwan et al., that revealed that KIR2DL2 was capable of mediating inhibitory signals even when the tyrosine residue in the ITIM region was mutated to phenylalanine. Further examination revealed that this was due to a co-receptor strategy requiring LILRB1, which encodes 4 canonical ITIMs in its CYT [282]. Antibody blocking studies with W6/32 revealed that although LILRB1 is insufficient to signal alone, it can signal in a KIR-dependent manner showcasing cooperation between inhibitory members of two distinct IgSF families. I found the same results with the blocking antibody, which suggests that LILRB1 may function with KIR2DL3 in a cooperative manner similar to what has been previously published for KIR2DL2. Another example of inhibitory receptor cooperation is observed with PIR-B, the mouse homolog to LILRB1, mediates inhibition of axonal

growth by associating with the p75 receptor [286]. With these findings I decided to switch my focus to optimize expression with yet another functional NK cell line.

My next approach utilized splenic mouse NK cells. These cells are stimulated with IL-2 and display a very potent killing response through ADCC [287]. I did not detect any inhibition of cytotoxicity by infected mouse NK cells when combined with the 721.221 cell line, which is MHC-I negative. Additionally, I did not observe any inhibition of cytotoxicity with NK cells infected with rVV encoding the empty vector, pSC66, or rVV-KIR/IpLITRΔCYT. Both KIR/IpLITR1.1b and 1.2a cytoplasmic regions inhibited cytotoxicity towards the .221-Cw3 cells at levels comparable with the control, KIR2DL3. Similar levels of inhibition by KIR2DL3 and the KIR/IpLITRs suggests that, in the context of this assay, the number of ITIM motifs did not have an effect on the strength of inhibition. KIR2DL3 and KIR/IpLITR1.2a contain 2 canonical ITIMs and KIR/IpLITR1.1b contains 2 canonical ITIMs and an ITSM. These results also suggest that an ITIM motif derived from either a fish or mammalian immunoregulatory protein receptor function at the same efficiency in the same system

I developed rVV encoding KIR/IpLITR1.1b TR and infected mouse NK cells resulting in expression of the chimeric proteins on the cell surface. Infected mouse NK cells expressing KIR/IpLITR1.1b TR were combined with either 721.221 or .221-Cw3 cells and measured for cytotoxicity. This was surprising because KIR/IpLITR1.1b TR does not contain canonical ITIM motifs and did not recruit either SHP-1 or SHP-2 in my previous immunoprecipitation experiments. After establishing that KIR/IpLITR1.1b and 1.2a are functional inhibitory receptors, I then directed my focus on the TM proximal CYT region of IpLITR1.1b. This region has three tyrosine residues but none are found in

the canonical ITIM sequence. The KIR/IpLITR1.1b distal CYT region is almost identical to the sequence of IpLITR1.2a and they most likely promote similar functional activities since they both recruit SHP-1 and SHP-2. However, the TM proximal CYT region of IpLITR1.1b is a unique stretch of sequence that does not recruit phosphatases so I was interested to determine if this region could elicit functional responses. The chimeric KIR/IpLITR1.1b TR that I developed contained the TM proximal region of KIR/IpLITR1.1b that lacked the canonical ITIMs found in the distal region (for clarification of this cytoplasmic region refer to the schematic diagram of KIR/IpLITR1.1b TR in Fig. 4.1.). As SHP recruitment is a common trait of inhibitory receptors, this result was surprising since I had previously found that KIR/IpLITR1.1b TR did not recruit zebrafish SHP-1 and SHP-2 while full-length 1.1b and 1.2a did (Chapter IV).

I did not have any success using chemical blockers of SHP-1 in order to investigate the potential involvement of SHP-1 in IpLITR-mediated inhibitory signaling. Sodium stibogluconate is a drug commonly used in the treatment of leishmaniasis and has also been shown to block SHP-1 function [288]. SSG did not have any effect on the inhibitory potential of the KIR/IpLITR chimeras, KIR2DL3, or LILRB1. Previously, SSG had been reported to block SHP-1 catalytic activity in *in vitro* studies [288]. The results of my work found that there was no difference in inhibition of cytotoxicity in the presence or absence of SSG. These findings are similar to those of authors who investigated the inhibitory role of the receptor LILRB4 with the use of SSG and also demonstrated that SSG had no effect [289]. The authors suggested that this was due to the possible involvement of phosphatases other than SHP-1, which may function in a

redundant manner [289]. In order to directly investigate the effect of SSG on SHP-1 mediated inhibition of cytotoxicity, I tested YTS cells transduced to express chimeric fusion proteins that consist of the EC and TM regions of KIR2DL1 fused to SHP-1 substituting for the CYT (YTS-KIR/SHP-1 WT) [73]. In addition to this, I also tested YTS cells transduced with the chimeric KIR/SHP-1 with a mutation in the catalytic region rendering the chimeric protein a trapping mutant. Previously, these transduced cells have been used to identify Vav1 as a substrate for SHP-1 in the regulation of NK cellular cytotoxicity [73]. By measuring the direct effects of SHP-1 on cellular cytotoxicity I could get a better idea if SSG was actually functioning as a SHP-1 inhibitor in cellular cytotoxicity. However, I found that various concentrations of SSG had no effect on YTS cellular cytotoxicity (Fig. 5.17). I decided to then look for another inhibitor that has been previously reported to inhibit SHP-1 activity. The PTP I inhibitor (chemical name: α-Bromo-4-hydroxyacetophenone, 4-hydroxyphenacyl Br) was used by Haga et al. to demonstrate that SHP-1 is involved in FcRL5-mediated inhibition of tyrosine phosphorylation events in human tonsillar memory B-cells [271]. However, when I tested the same drug, I observed quite different results. At low concentrations (2-20 μM) there was no measurable killing response from the YTS cells and at higher concentrations (200-2000 μM) it appeared that cytotoxicity of YTS was reverted to levels higher than before but viability staining uncovered that all cells in the assay at these concentrations of PTP I inhibitor were dead (effectors and targets) (Fig. 5.19). Similar results were observed with YTS cells transfected with KIR/IpLITR1.1b TR and KIR/IpLITRΔCYT (Fig. 5.19). Although the reason for the toxicity of target and effector cells is unknown, the discrepancy between the duration of time to measure tyrosine

phosphorylation (i.e. seconds to minutes) versus ^{51}Cr release assays (i.e. hours) may have something to do with the reason why this drug treatment gave different results in my experiments and the studies of Haga et al. [271].

The final approach that was undertaken to examine the role of SHP-1 in IpLITR-mediated inhibition was the introduction of a catalytically-inactive DN-SHP-1 in the cytotoxicity assays. DN-SHP-1 binds to the CYT ITIM regions competitively blocking the sites from recruiting functional inhibitory enzymes. This is a common approach that has been used to examine the role of SHP-1 in many situations of cellular signaling inculding the regulation of DC function [290] and its role in NK effector responses [56]. Expression of the DN-SHP-1 significantly reversed the inhibitory function of pSC66-KIR2DL3, confirming that SHP-1 is required for KIR2DL3-mediated inhibition of NK cell killing responses. When I examined the SHP-1 dependency of the two inhibitory IpLITR-types, only KIR/IpLITR1.2a was sensitive to the presence of the DN-SHP-1, indicating that although IpLITR1.1b binds SHP-1, its inhibitory activity is not completely dependent on this phosphatase. Furthermore, the CYT of this receptor encodes a unique tyrosine-containing membrane proximal region that is not present in IpLITR1.2a and does not appear to bind SHP-1 or SHP-2, as suggested by the data presented in chapter III. An important caveat to the approach of using dominant negative proteins is that the observed effects may in part be due to the fact that the DN-SHP-1 is not only competitively blocking SHP-1 but all other molecules that may be recruited to this region. With this in mind, I cannot rule out the possibility that other inhibitory molecules, which possess functional redundancy with SHP-1, may also be involved.

The results presented in this chapter are the first evidence of SHP-1 mediated inhibition of cytotoxicity by IpLITR receptors. Combining the results of this chapter with those of chapter IV, it is now evident that IpLITR1.1b and IpLITR1.2a are indeed bona fide inhibitory immunoregulatory receptors. This work was the first demonstration of the functional relationship between inhibitory immune receptors, specifically IpLITRs, and SHP-1 in teleosts. These findings reinforce that the strategy of inhibition of leukocyte effector responses through the use of tyrosine phosphatases is an ancient and conserved strategy among vertebrates. Interestingly, the results from these experiments also suggest that there is a SHP-1-independent mechanism of inhibition originating in the TM proximal region of IpLITR1.1b but absent in IpLITR1.2a and the further examination of how this response may be occurring is the focus of the next chapter.

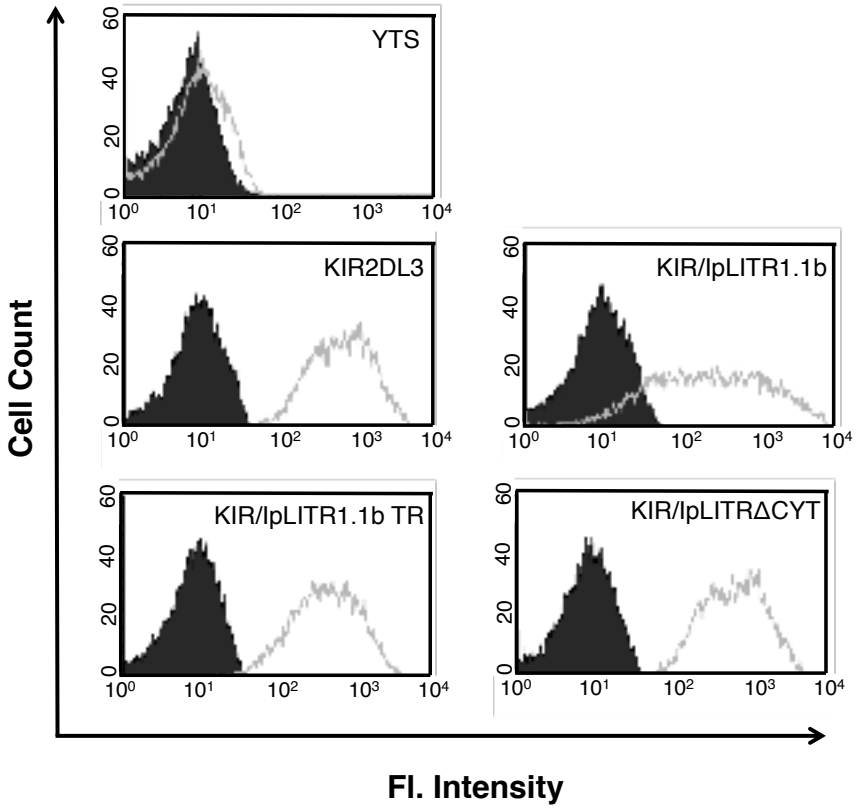

Figure 5.1. Surface expression of KIR2DL3 and KIR/IpLITR Chimeric receptors in transduced YTS cells. Flow cytometry analysis of YTS cells transduced with KIR2DL3 or the chimeric constructs KIR/IpLITR1.1b, KIR/IpLITR1.1b TR, or KIR/IpLITRΔCYT and bulk-sorted following puromycin selection. Cells were stained with the anti-KIR2D antibody DX27 and a goat anti-mouse IgG coupled to PE. The open histogram indicates staining with DX27 while the shaded histogram represents cells stained with an IgG2a isotype control. Results are representative of at least three independent experiments.

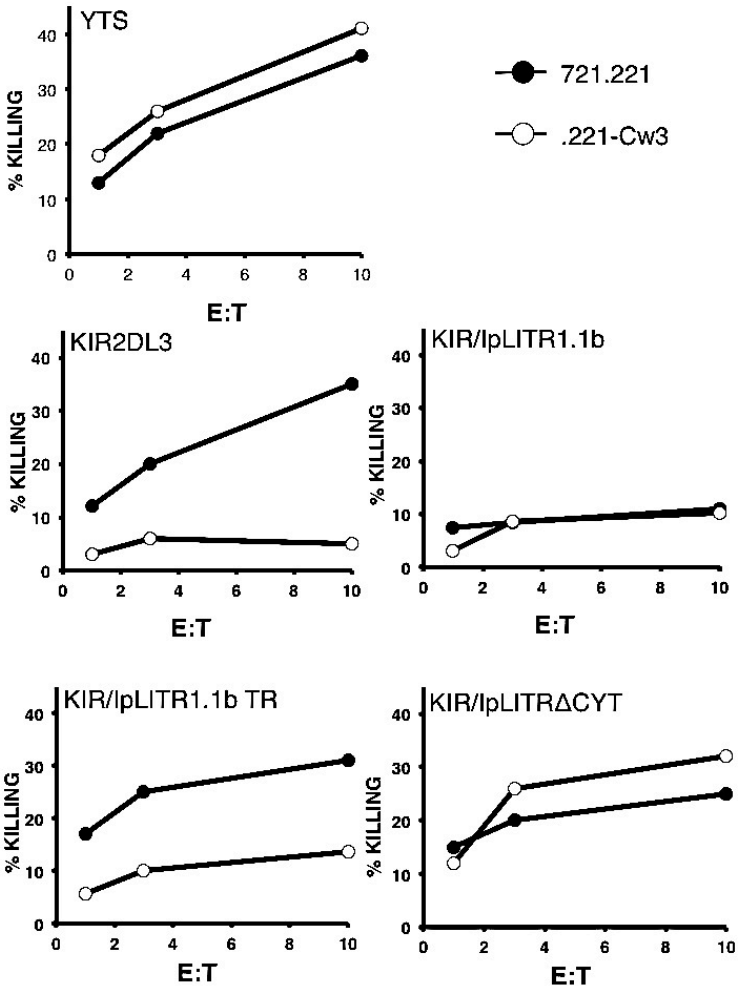

Figure 5.2. KIR2DL3 and KIR/IpLITR1.1b TR, but not KIR/IpLITR1.1b, mediate inhibition of cytotoxicity. rVV-infected YTS cells were combined with ^{51}Cr-loaded 721.221 cells or .221-Cw3 cells at effector-to-target (E:T) ratios of 1:1, 3:1, and 10:1. Cells were combined for 4 hours and specific lysis was measured by ^{51}Cr release in supernatant. ^{51}Cr release was calculated as: % lysis = 100 X (mean sample release - mean spontaneous release)/(mean total release - mean spontaneous release) and is represented as % killing. Data presented is representative of at least three independent experiments.

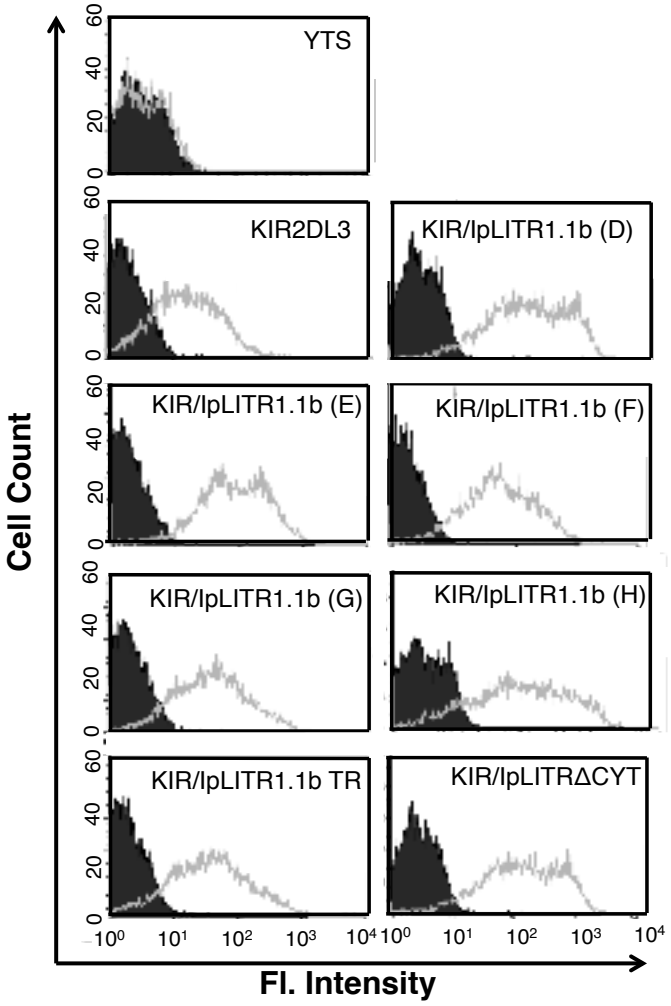

Figure 5.3. Surface expression of KIR2DL3 and KIR/IpLITR Chimeric receptors in transfected YTS subclones. Flow cytometric analysis of YTS cells transduced with KIR2DL3 or KIR/IpLITR chimeras. All transduced YTS cells were bulk sorted following transduction and puromycin selection. YTS transduced with KIR/IpLITR1.1b were then sub-cloned following bulk sorting. The letters 'D', 'E', 'F', 'G', and 'H' all refer to specific sub-clones of IpLITR1.1b-transduced YTS cells. The empty grey histogram indicates staining with the anti-KIR antibody DX27 followed by goat anti-mouse IgG-PE while the shaded histogram represents cells stained with an isotype control.

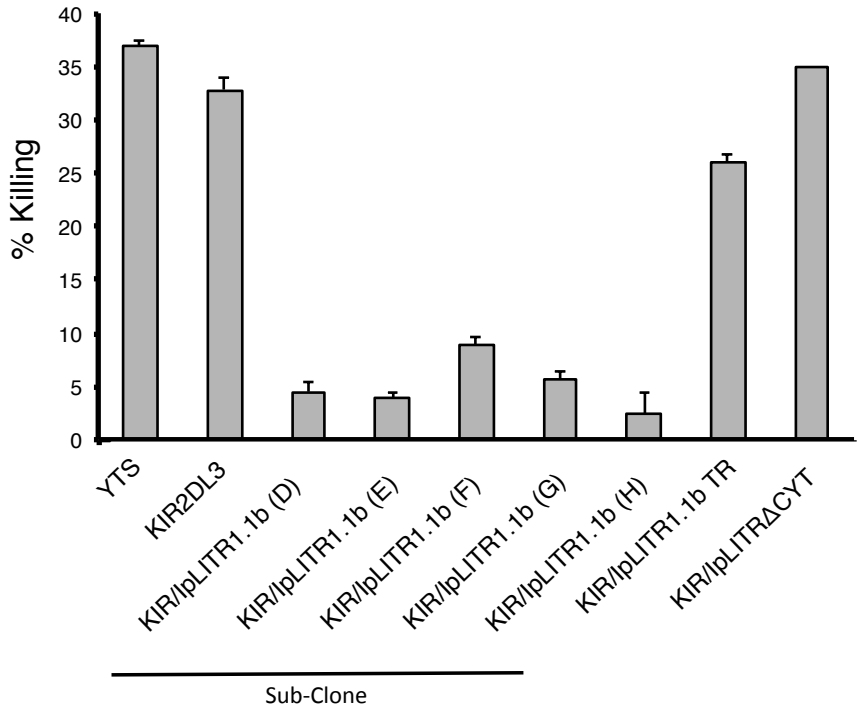

Figure 5.4. Subcloned YTS transduced with KIR/IpLITR1.1b did not demonstrate strong killing of 721.221 cells. Transduced and non-transduced YTS cells were combined with ^{51}Cr-loaded 721.221 cells at effector:target (E:T) ratio at 10:1. The letters 'D', 'E', 'F', 'G', and 'H' all refer to specific sub-clones of IpLITR1.1b-transduced YTS cells. Cells were combined for 4 hours and specific lysis was measured by ^{51}Cr release in supernatant. ^{51}Cr release was calculated as: % lysis = 100 X (mean sample release - mean spontaneous release)/(mean total release - mean spontaneous release) and is represented as % killing. Each bar represents the Mean ± SEM. Data presented is representative of at least two independent experiments.

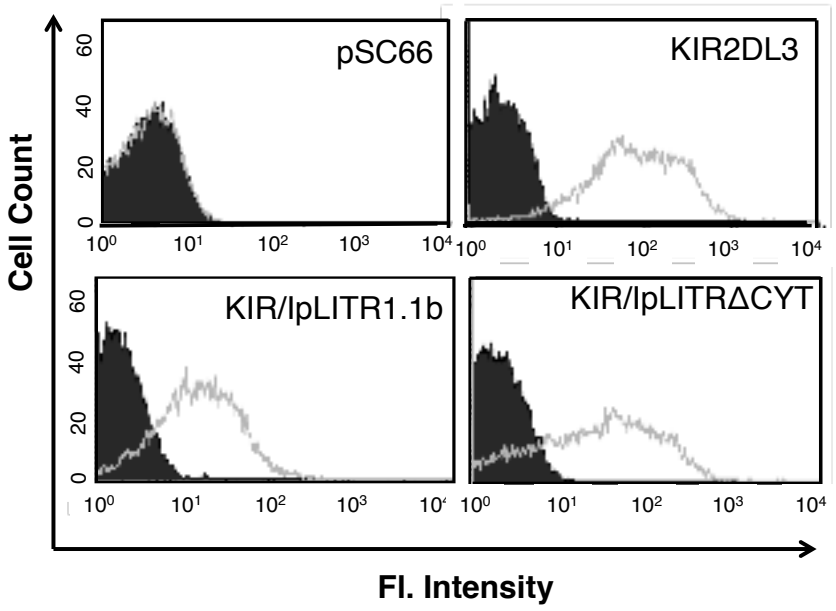

Figure 5.5. Surface expression of KIR2DL3, KIR/IpLITR1.1b, and KIR/IpLITRΔCYT in NK92 cells. Flow cytometric analysis of surface expression of KIR/IpLITR chimeras and KIR2DL3 after 4 hours infection with rVV encoding KIR2DL3, KIR/IpLITR1.1b, KIR/IpLITRΔCYT, or the empty vector, pSC66. NK92 cells were infected at an MOI of 20. Cells were stained with the anti-KIR2D mAb, DX27 and followed with a goat anti-mouse IgG-PE antibody (as indicated by the empty histogram. The shaded histogram indicates staining with an IgG2a isotype control antibody.

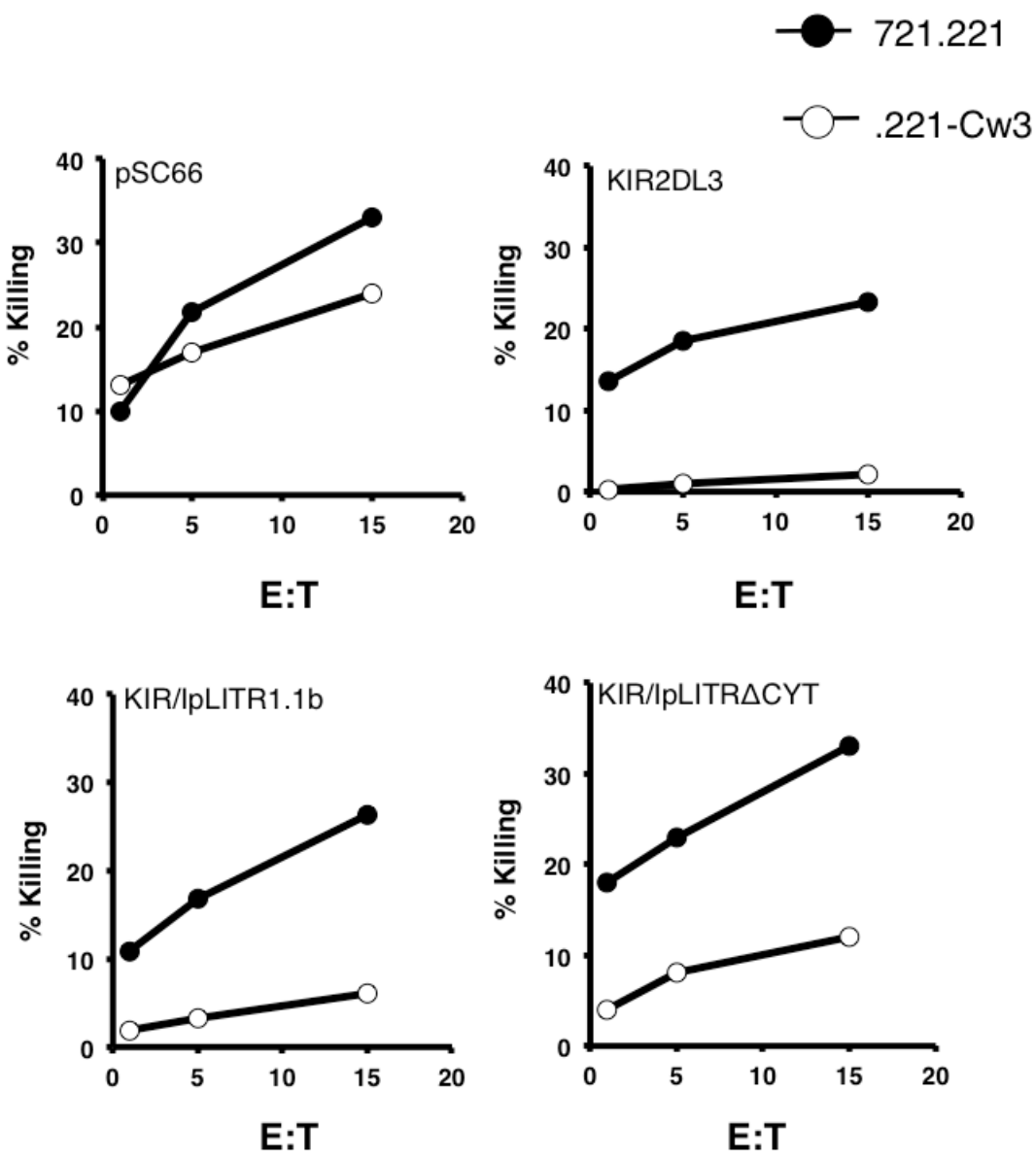

Figure 5.6. Inhibition of target cell killing by NK92 cells expressing KIR2DL3, KIR/IpLITR1.1b, and KIR/IpLITRΔCYT. NK92 cells were infected for 4 hours with rVV prior to mixing with either 721.221 cells (black circles) or .221-Cw3 (white circles), at E:T ratios of 1:1, 5:1, and 15:1. Effectors and targets were then incubated for a further 4 h and target cell killing was measured from cellular supernatants using the ^{51}Cr release assay. ^{51}Cr release was calculated as: % lysis = 100 X (mean sample release - mean spontaneous release)/(mean total release - mean spontaneous release) and is represented as % killing. Data presented is a representative from two independent experiments.

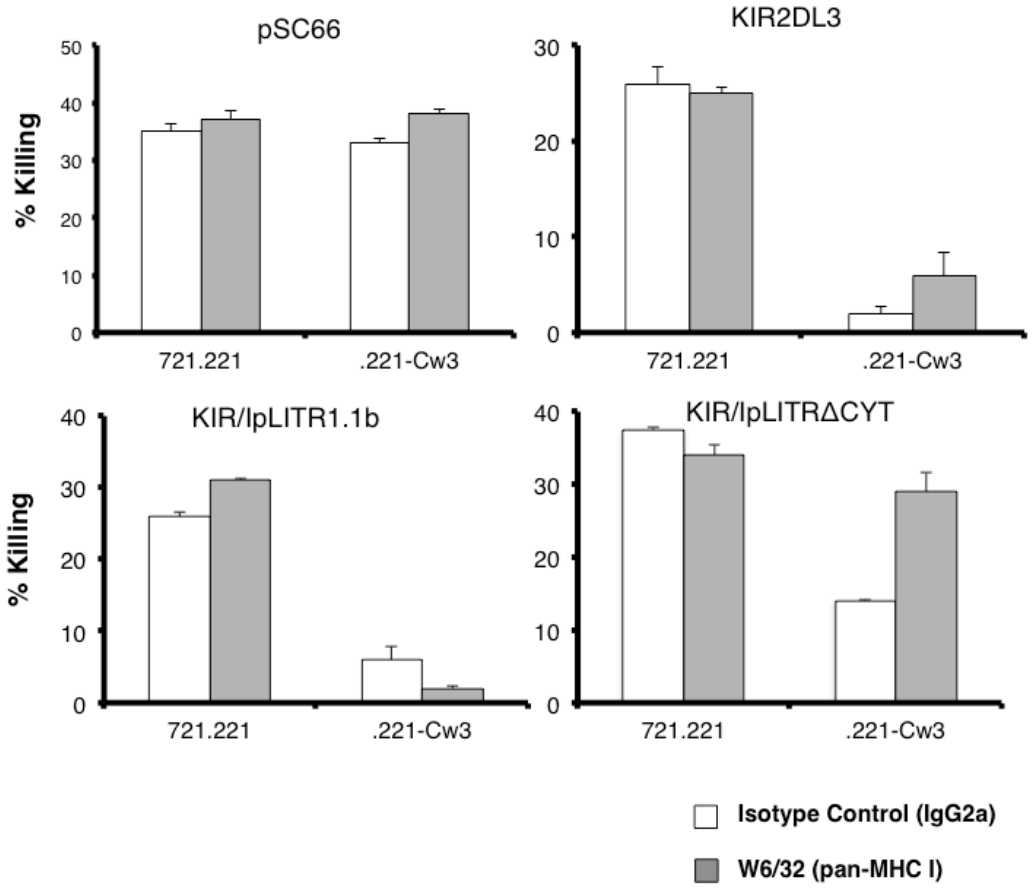

Figure 5.7. Inhibitory signaling by KIR/IpLITRΔCYT inhibition is reverted by the pan-MHC I mAb W6/32. NK92 cells were infected with rVV encoding an empty pSC66 vector, wild-type KIR2DL3 or the chimeric receptors KIR/IpLITR1.1b, or KIR/IpLITRΔCYT at an MOI of 20. Infected NK92 cells were incubated in the presence of an IgG2a isotype control mAb (white bars) the pan-MHC I mAb W6/32 (grey bars) at a final concentration of 10 µg/ mL for 30 min prior to performing the cytotoxicity assay. The target cells used were 721.221 cells or .221-Cw3 and effectors and targets were incubated at an E:T of 10:1 for 4 h prior to performing the ^{51}Cr release assay. ^{51}Cr release was calculated as: % lysis = 100 X (mean sample release - mean spontaneous release)/(mean total release - mean spontaneous release) and is represented as % killing on the y-axis of each graph. Each bar represents the Mean ± SEM. Data presented is representative of two independent experiments.

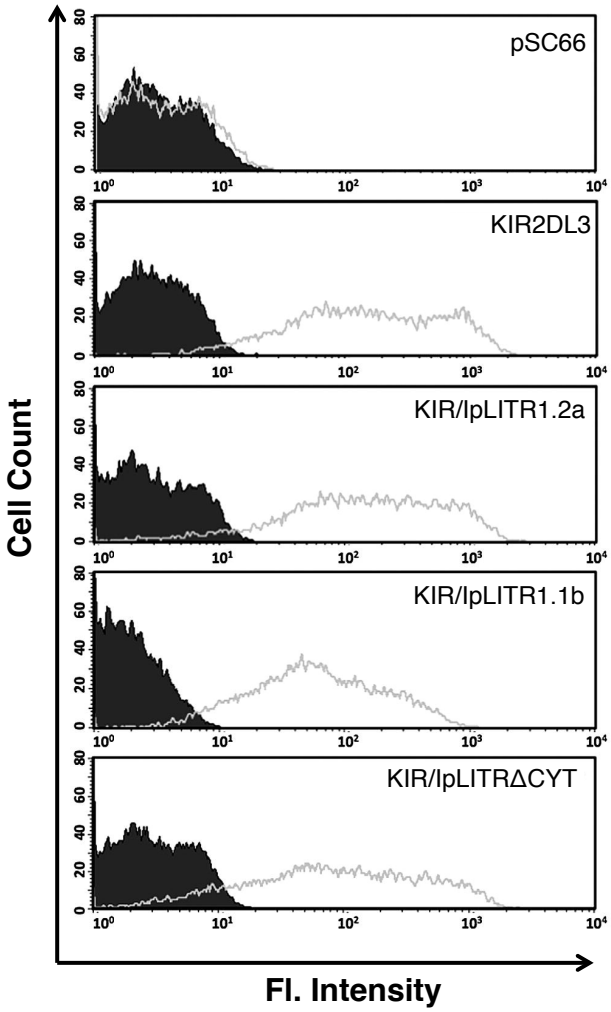

Figure 5.8. Surface expression of KIR2DL3 and KIR/IpLITR Chimeric receptors in transfected NK cells. Mouse NK cells were infected with rVV-WR (MOI of 20) encoding an empty pSC66 vector, wild-type KIR2DL3 or the chimeric receptors KIR/IpLITR1.2a, KIR/IpLITR1.1b, and KIR/IpLITRΔCYT. The surface expression of each receptor was determined by flow cytometry using the anti-KIR mAb DX27 followed by staining with a goat anti-mouse IgG-PE. The secondary pAb alone control is indicated by the solid histogram and the anti-KIR2DL3 staining with DX27 is indicated by the empty histogram.

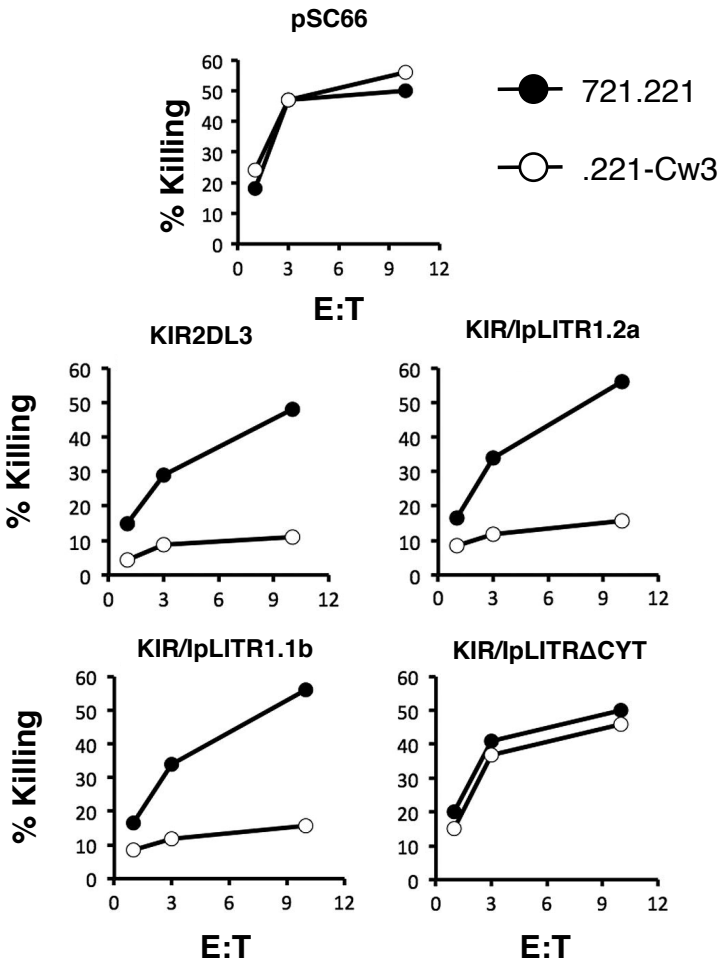

Figure 5.9. Inhibition of NK cell-mediated cellular cytotoxicity by KIR/IpLITR chimeras. Mouse NK cells were infected for 4 h with rVV prior to mixing them with either 721.221 cells (black circles) or .221-Cw3 (white circles), both target cell lines coated with the anti-MHC class II antibody L243 at 1μg/ml, at effector-to-target (E:T) ratios of 1:1, 3:1, and 10:1. Effectors and targets were then incubated for 4 hours and target cell killing was measured from cellular supernatants using the ^{51}Cr release assay. ^{51}Cr release was calculated as: % Killing = 100 X (mean sample release - mean spontaneous release)/(mean total release - mean spontaneous release). Data presented is a representative from three independent experiments.

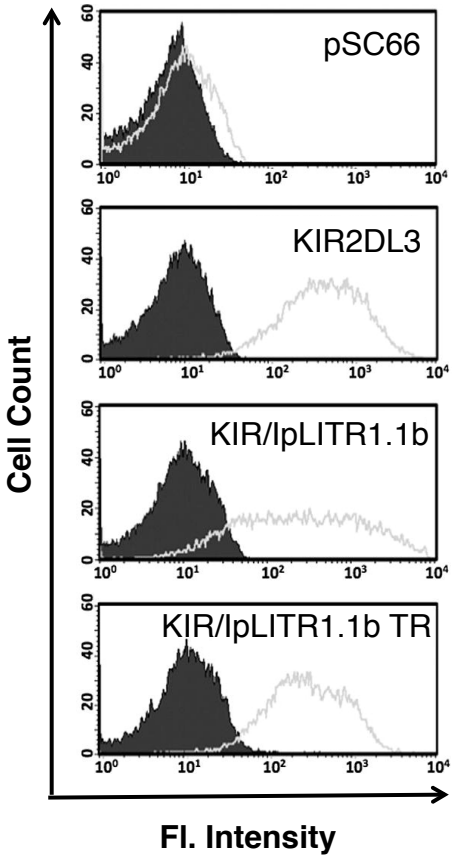

Figure 5.10. Surface expression of KIR2DL3, KIR/IpLITR1.1b, and KIR/IpLITR1.1b TR in NK cells. Mouse NK cells were infected with rVV-WR (MOI of 20) encoding an empty pSC66 vector, wild-type KIR2DL3 or the chimeric receptors KIR/ IpLITR1.1b, and KIR/IpLITR1.1b TR. The surface expression of each receptor was determined by flow cytometry using the anti-KIR mAb DX27 followed by staining with a secondary goat anti-mouse IgG-PE. The secondary pAb alone control is indicated by the solid histogram and the anti-KIR2DL3 staining with DX27 is indicated by the empty histogram.

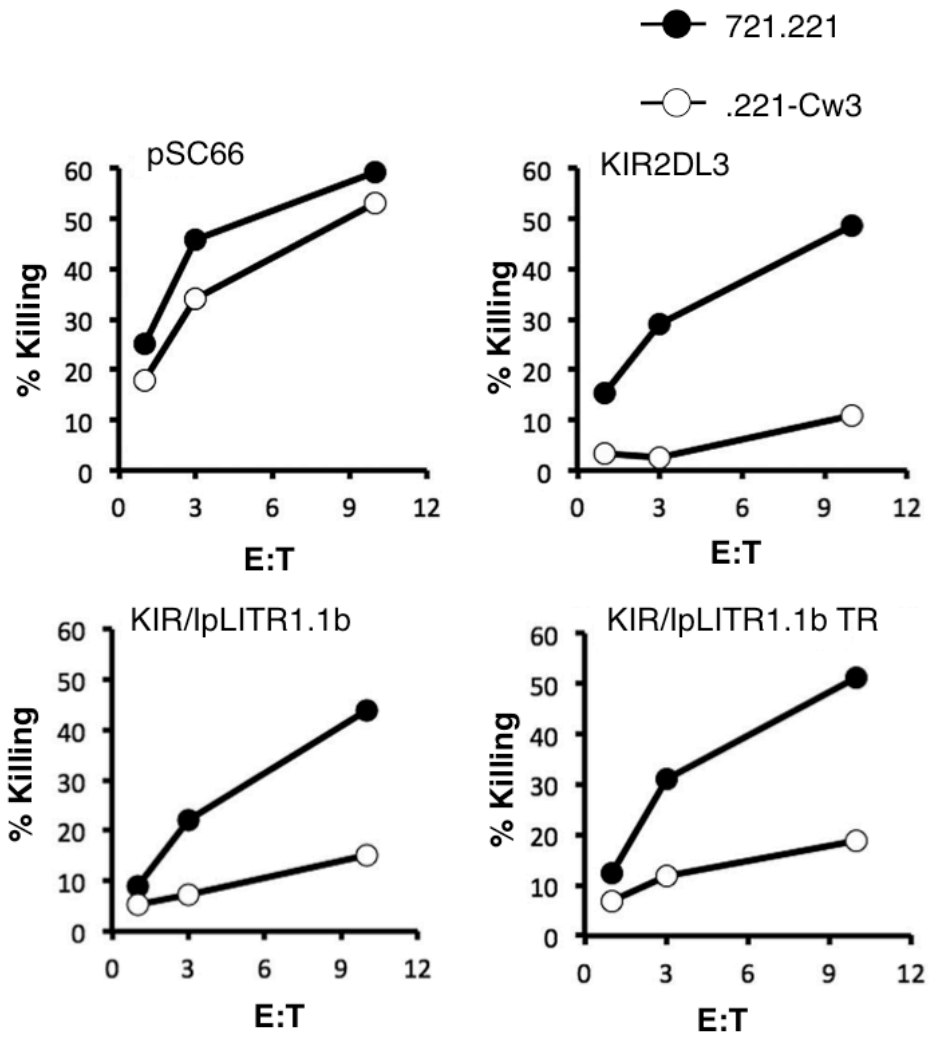

Figure 5.11. The membrane proximal CYT region of IpLITR1.1b inhibits NK cell-mediated cellular cytotoxicity. Mouse NK cells were infected for 4 h with rVV prior to mixing them with either 721.221 cells (black circles) or .221-Cw3 (white circles) at E:T ratios of 1:1, 3:1, and 10:1. Effectors and targets were then incubated together for 4 hours and target cell killing was measured from cellular supernatants using the ^{51}Cr release assay. ^{51}Cr release was calculated as: % lysis = 100 X (mean sample release - mean spontaneous release)/(mean total release - mean spontaneous release). Data presented is representative from three independent experiments.

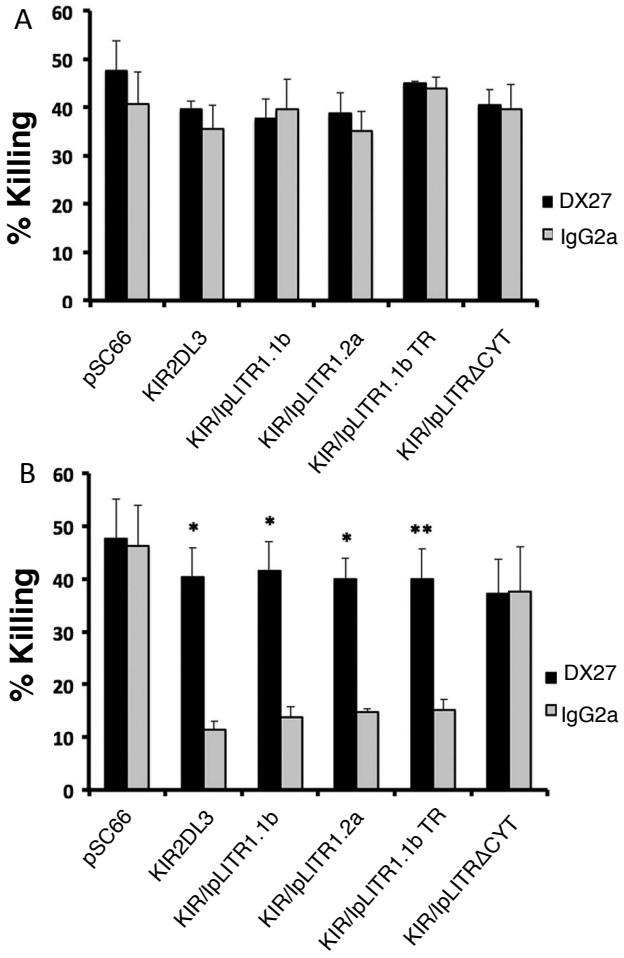

Figure 5.12. Inhibition of cytotoxicity in NK cells is due to specific interactions between KIR/IpLITR chimeras with the ligand HLA-Cw3. Mouse NK cells were infected with rVV encoding an empty pSC66 vector, KIR2DL3 or the chimeric receptors KIR/IpLITR1.2a, KIR/IpLITR1.1b, KIR/IpLITR1.1b TR, KIR/IpLITRΔCYT (MOI of 20). Infected NK cells were incubated in the presence of an IgG2a isotype control mAb (grey bars) or the anti-KIR2D mAb DX27 (black bars) at a final concentration of 10 µg/ mL for 30 min prior to performing the cytotoxicity assay. The target cells used were (A) 721.221 cells or (B) .221-Cw3 and effectors and targets were incubated at an E:T of 10:1 for 4 h prior to performing the ^{51}Cr release assay. ^{51}Cr release was calculated as: % lysis = 100 X (mean sample release - mean spontaneous release)/(mean total release - mean spontaneous release). Data presented is the pooled results from three independent experiments. Each bar represents the mean ± SEM. (*$p < 0.01$ and **$p < 0.05$ when comparing IgG2a isotype (grey bars) to anti-DX27 mAb (black bars) for each construct tested).

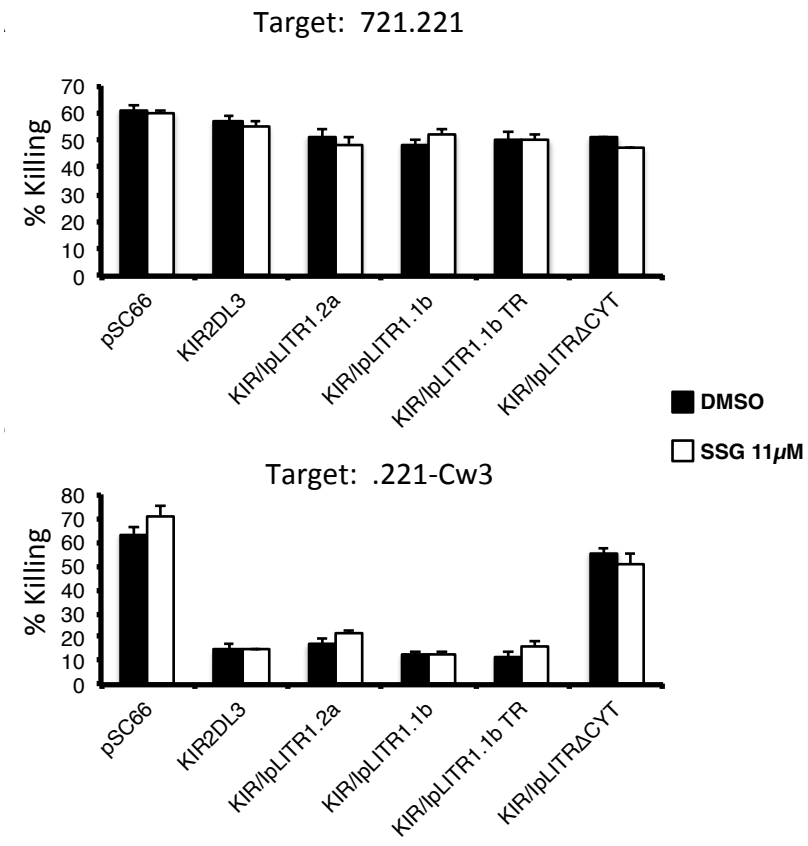

Figure 5.13. Sodium stibogluconate does not affect inhibitory signaling by KIR/IpLITR chimeric proteins. Mouse NK cells were infected with rVV encoding the empty vector pSC66, KIR2DL3, KIR/IpLITR1.2a, KIR/IpLITR1.1b, KIR/IpLITR1.1b TR, or KIR/IpLITRΔCYT for 4 hours. Following infection, cells were treated with the SHP-1 inhibitor sodium stibogluconate (SSG) at 11μM for 30 min. Prior to the assay, the target cells, 721.221 and 721.221 expressing HLA-Cw3, were incubated with the anti-MHC class II antibody L243 at 1μg/ml for 30 minutes. The assay was conducted at an effector-to-target (E:T) ratio of 10:1. Following incubation for 4 hours, supernatant was measured for ^{51}Cr release. ^{51}Cr release was calculated as: % Killing = 100 X (mean sample release - mean spontaneous release)/(mean total release - mean spontaneous release). Data presented is representative of two independent experiments. Each bar represents the mean ± SEM.

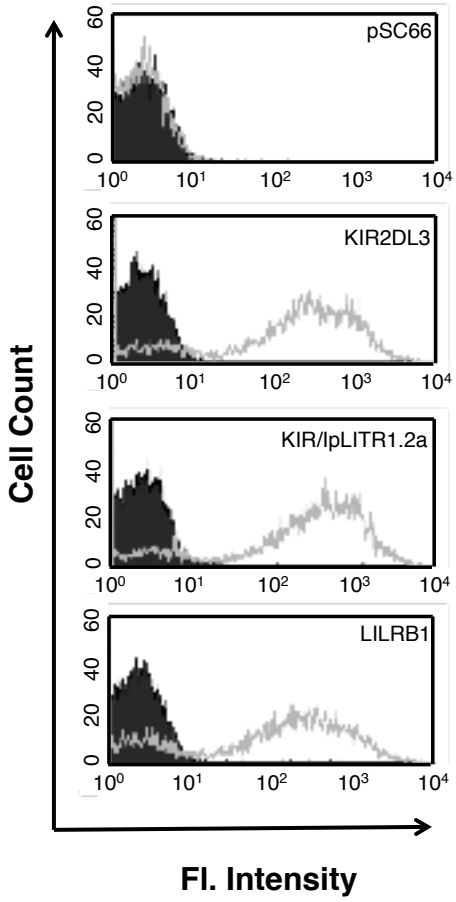

Figure 5.14. Surface expression of LILRB1 on mouse NK cells. Mouse NK cells were infected with rVV encoding the empty vector pSC66, KIR2DL3, KIR/IpLITR1.2a, KIR/IpLITR1.1b, KIR/IpLITR1.1b TR, or KIR/IpLITRΔCYT for 4 hours. Following infection, cells were stained with that anti-KIR2D mAb DX27 or an anti-LILRB1 mAb (empty histogram). The shaded histogram represents cells stained with PE-goat anti-mouse IgG pAb alone.

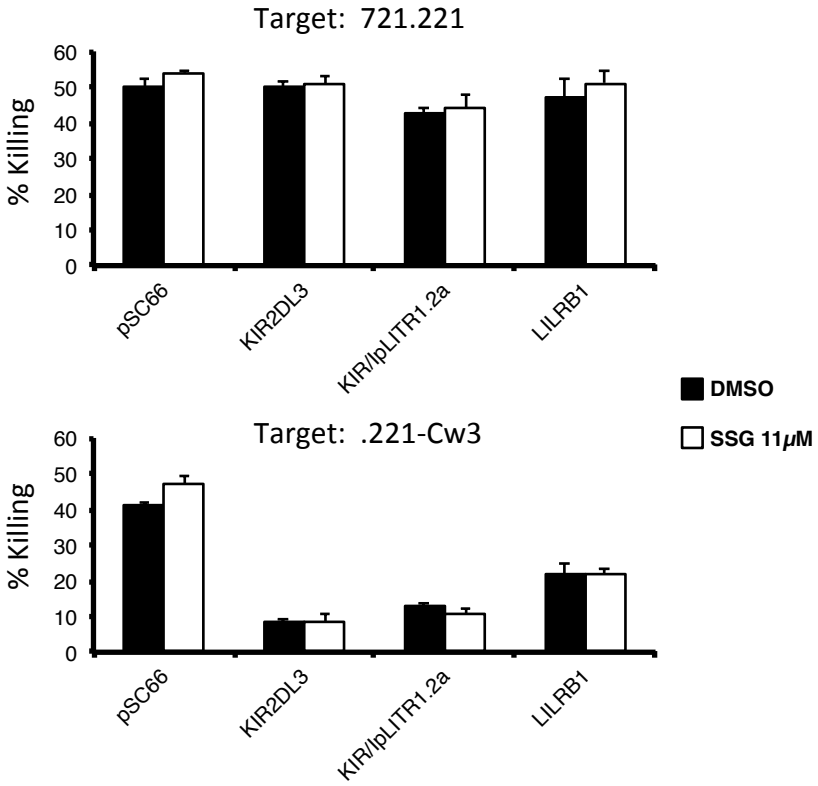

Figure 5.15. Sodium stibogluconate does not affect inhibitory signaling by LILRB1. **Mouse** NK cells were infected with rVV encoding the empty vector pSC66, KIR2DL3, KIR/IpLITR1.2a, KIR/IpLITR1.1b, KIR/IpLITR1.1b TR, or KIR/IpLITRΔCYT for 4 hours. Following infection, cells were treated with the SHP-1 inhibitor sodium stibogluconate (SSG) at 11μM for 30 min. Prior to the assay, the target cells, 721.221 and 721.221 expressing HLA-Cw3, were incubated with the anti-MHC class II antibody L243 at 1μg/ml for 30 minutes. The assay was conducted at an effector-to-target (E:T) ratio of 10:1. Following incubation for 4 hours, supernatant was measured for ^{51}Cr release. ^{51}Cr release was calculated as: % Killing = 100 X (mean sample release - mean spontaneous release)/(mean total release - mean spontaneous release). Data presented is representative of two independent experiments. Each bar represents the mean ± SEM.

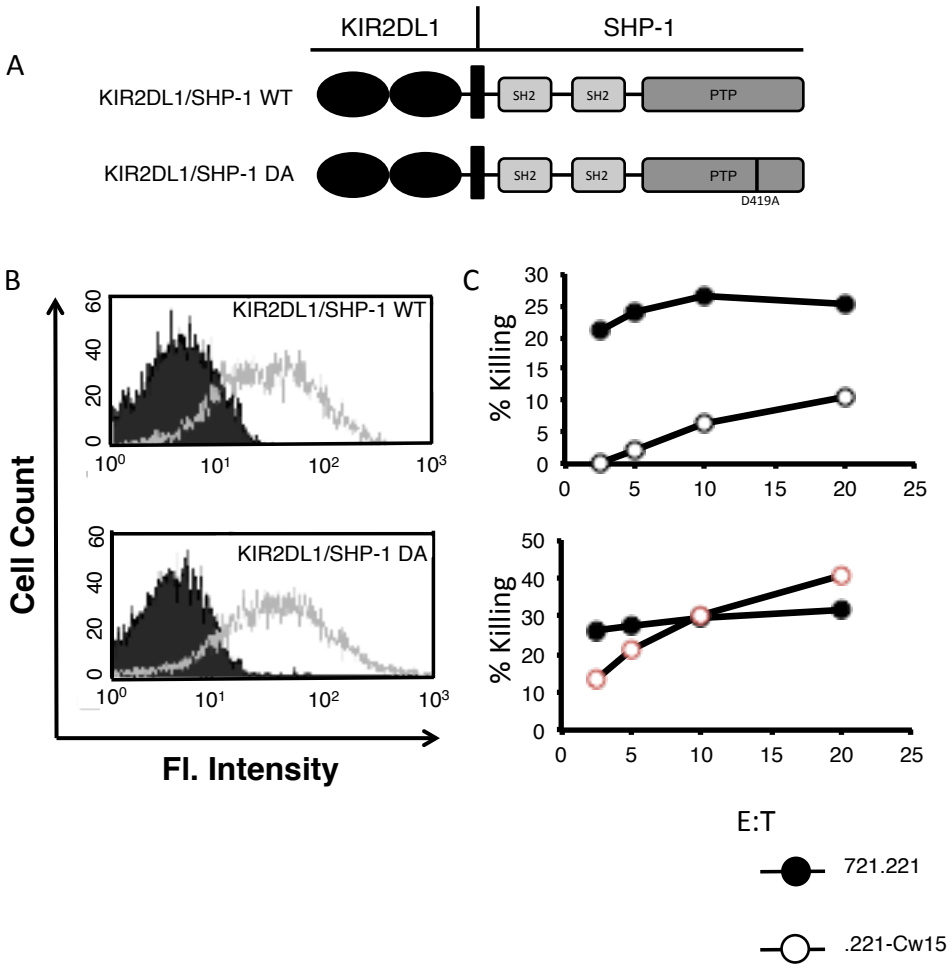

Figure 5.16. Expression and function of chimeric 2DL1/SHP-1 molecules in YTS cells. (A) Schematic representation of Chimeric 2DL1/SHP-1 molecules. (B) Staining of YTS cells expressing these chimeric constructs. Shaded profiles represent PE-labeled goat anti-mouse Ab only. Dark lines represent staining with the anti-2DL1 mAb EB6 followed by staining with a PE-conjugated goat anti-mouse IgG Ab. (C) Lysis of 721.221 (black circles) or 221-Cw15 (white circles) by YTS-2DL1/SHP-1(WT) (top graph) and YTS-2DL1/SHP-1(DA) (bottom graph) cells at the effector-to-target ratios (E:T) of 2.5:1, 5:1, 10:1, and 20:1. Results are representative of two independent experiments.

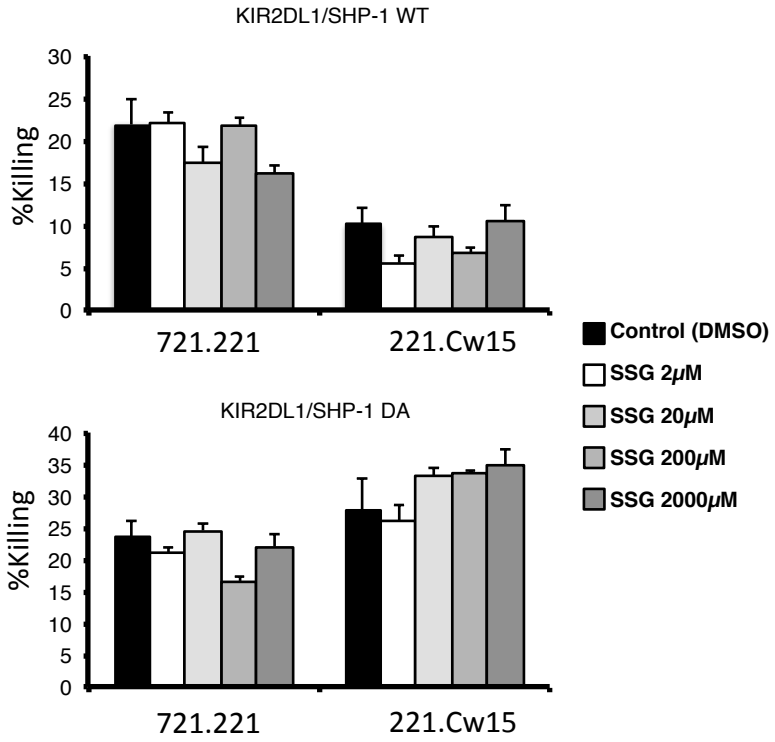

Figure 5.17. Sodium stibogluconate does not affect inhibitory signaling by KIR2DL1/SHP-1 chimeric proteins. YTS cells transduced with either KIR2DL1/SHP-1 WT or KIR2DL1/SHP-1 DA were treated with the SHP-1 inhibitor sodium stibogluconate (SSG) for 30 min at the concentrations indicated prior to mixing with either 721.221 or 221.Cw15 at an effector-to-target (E:T) ratio of 10:1. Following incubation of 4 hours, supernatant was measured for ^{51}Cr release. ^{51}Cr release was calculated as: % Killing = 100 X (mean sample release - mean spontaneous release)/(mean total release - mean spontaneous release). Data presented is representative of two independent experiments. Each bar represents Mean ± SEM.

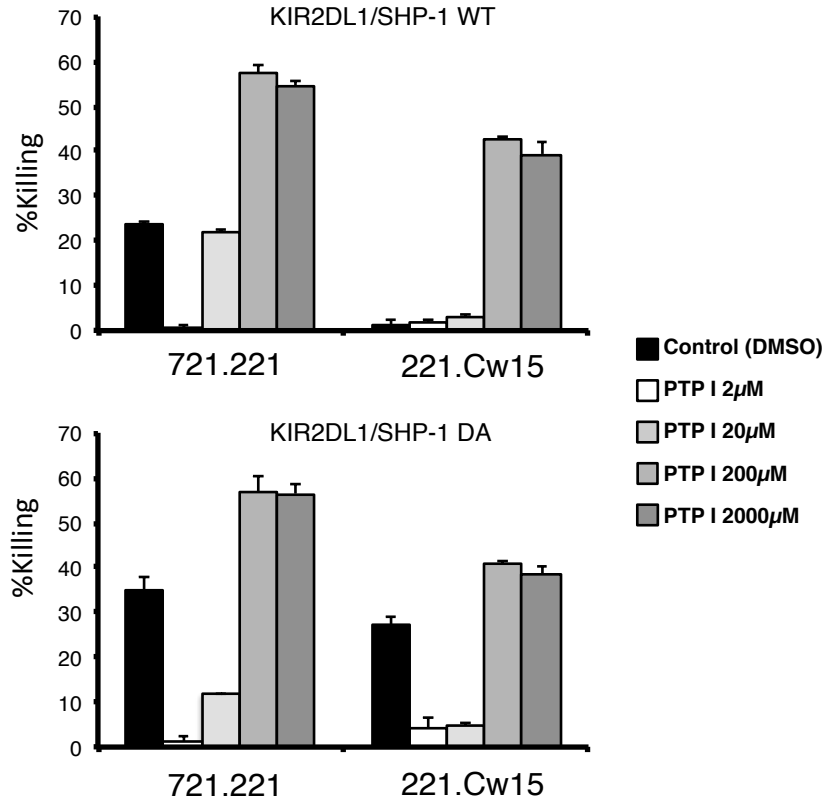

Figure 5.18. PTP Inhibitor I does not specifically revert inhibitory signaling by KIR2DL1/SHP-1 chimeric proteins. YTS cells transduced with either KIR2DL1/SHP-1 WT or KIR2DL1/SHP-1 DA were treated with the SHP-1 inhibitor PTP Inhibitor I for 30 min at the concentrations indicated prior to mixing with either 721.221 or 221.Cw15 at an effector-to-target (E:T) ratio of 10:1. Following incubation of 4 hours, supernatant was measured for ^{51}Cr release. ^{51}Cr release was calculated as: % Killing = 100 X (mean sample release - mean spontaneous release)/(mean total release - mean spontaneous release). Data presented is representative of two independent experiments. Each bar represents the mean ± SEM.

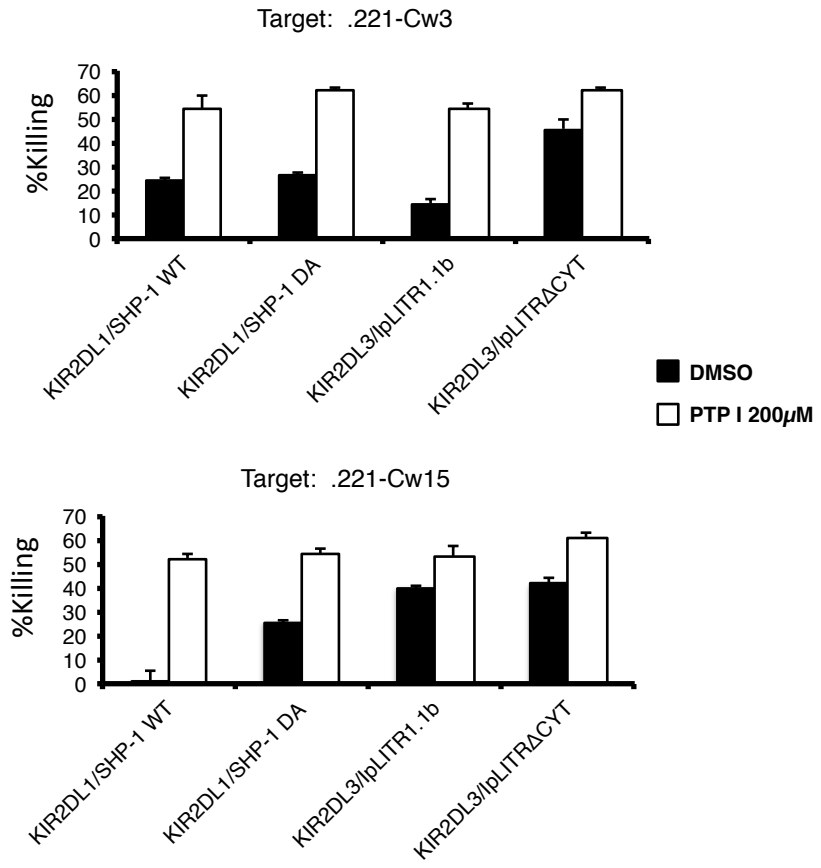

Figure 5.19. PTP Inhibitor I does not affect inhibitory signaling by KIR2DL1/SHP-1 chimeric proteins. YTS cells transduced with either KIR2DL1/SHP-1 WT, KIR2DL1/SHP-1 DA, KIR/IpLITR1.1b TR or KIR/IpLITRΔCYT were treated with the SHP-1 inhibitor PTP Inhibitor I at for 30 min at the concentrations indicated prior to mixing with either 221-Cw3 or 221.Cw15 at an effector-to-target (E:T) ratio of 10:1. Following incubation of 4 hours, supernatant was measured for ^{51}Cr release. ^{51}Cr release was calculated as: % Killing = 100 X (mean sample release - mean spontaneous release)/(mean total release - mean spontaneous release). Data presented is representative of at least two independent experiments. Each bar represents the mean ± SEM.

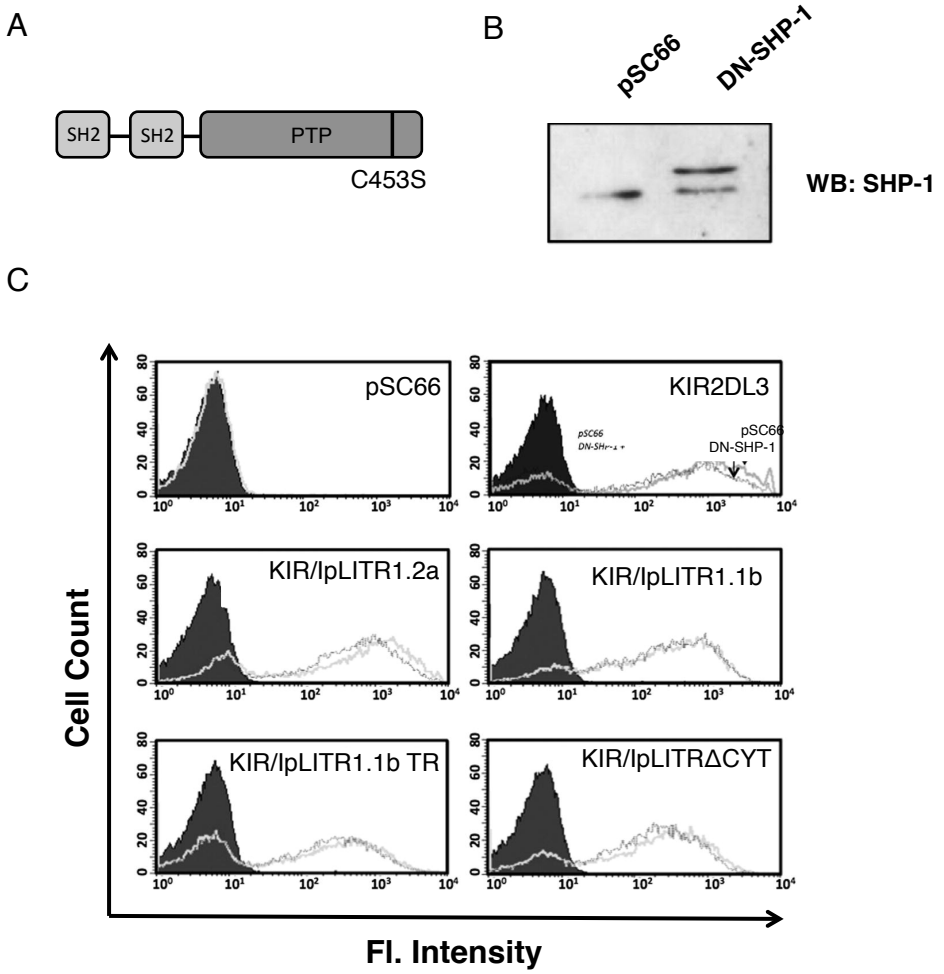

Figure 5.20. Schematic representation, WB detection, and cell surface expression of DN-SHP-1 in mouse NK cells. (A) Schematic diagram of the DN-SHP-1 rendered catalytically inactive by a C453S mutation. B) Western blot analysis indicating DN-SHP-1 expression in the lysates of rVV-infected cells. The pSC66 lane indicates NK infected with rVV encoding the empty vector, pSC66, the DN-SHP-1. C) NK cells were co-infected with rVV-WR (MOI of 20) encoding an empty pSC66 vector or pSC66-DN-SHP-1 in conjunction with wild-type KIR2DL3 or the chimeric receptors KIR/IpLITR1.2a, KIR/IpLITR1.1b, KIR/IpLITR1.1b TR and KIR/IpLITRΔCYT. The surface expression of each receptor co-expressed with pSC66 or DN-SHP-1 was determined by flow cytometry using the anti-KIR mAb DX27 followed by staining with a PE-goat anti-mouse IgG pAb . The secondary pAb alone control is indicated by the solid histogram and the anti-KIR2DL3 staining with DX27 for pSC66 or DN-SHP-1 co-expressed with the different receptors is indicated by the empty histograms.

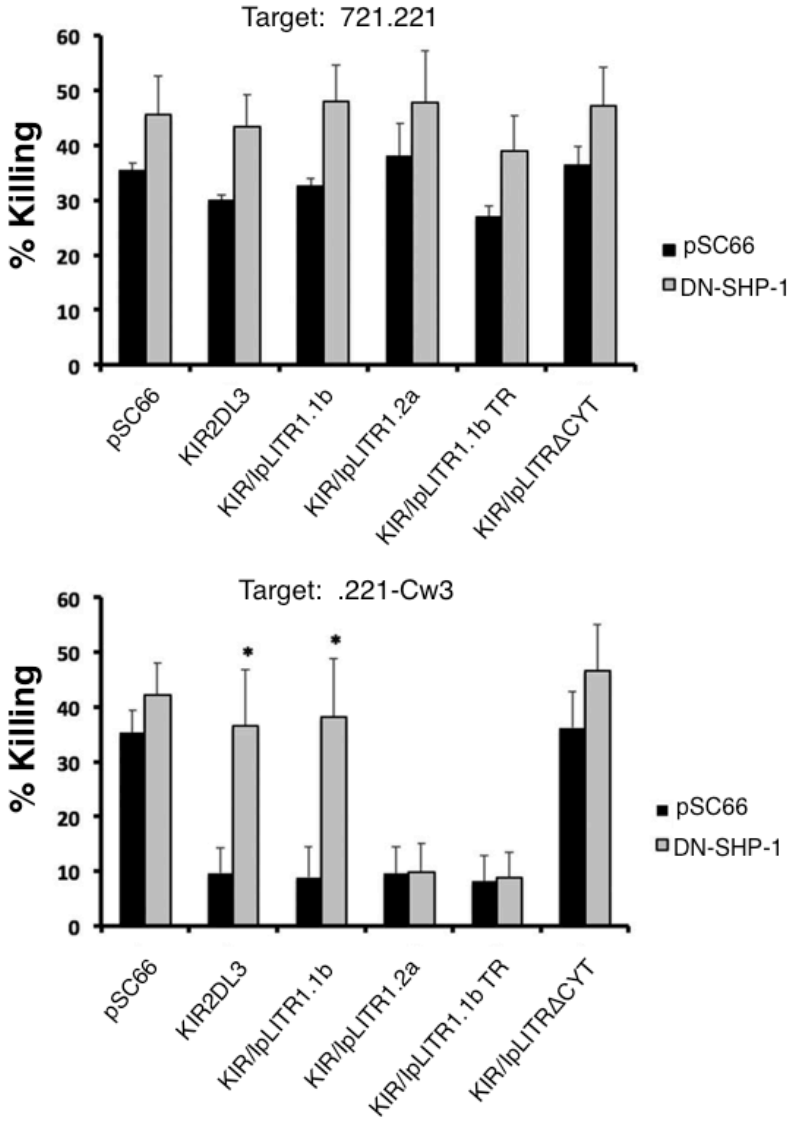

Figure 5.21. Catalytically inactive SHP-1 abrogates IpLITR1.2a but not IpLITR1.1b-mediated inhibitory signaling in mouse NK cells. Killing assays were performed with NK cells expressing the indicated various receptors co-infected with either DN-SHP-1 (grey bars) or rVV-pSC66 (black bars). The target cells used were 721.221 cells or 721.221 expressing HLA-Cw3. Prior to the assay, the target cells were incubated with the anti-MHC class II antibody L243 at 1μg/ml for 30 minutes. The assay was conducted at an effector-to-target (E:T) ratio of 10:1 for 4 h prior to performing the ^{51}Cr release assay. ^{51}Cr release was calculated as: % Killing = 100 X (mean sample release - mean spontaneous release)/(mean total release - mean spontaneous release). Data presented is the pooled results from two independent experiments. Each bar represents the mean ± SEM. (*$p < 0.05$ and **$p < 0.05$ when comparing DN-SHP-1 (grey bars) to pSC66 (black bars) for each construct tested).

CHAPTER VI

CHARACTERIZATION OF A PHOSPHATASE-INDEPENDENT INHIBITORY SIGNALING PATHWAY INITIATED BY IpLITR1.1b

Portions of this chapter have been previously published in:
Montgomery BCS, Cortes, Burshtyn DN, and Stafford JL. 2012. Channel catfish leukocyte immune-type receptor mediated inhibition of cellular cytotoxicity is facilitated by SHP-1-dependent and –independent mechanisms. *Developmental and Comparative Immunology*, 37(1): 151-63.

6.1 Introduction

In the previous chapter I demonstrated that the CYT regions of IpLITR1.2a and IpLITR1.1b could promote signaling events resulting in the inhibition of NK cell killing. These findings reinforce that IpLITR1.1b and IpLITR1.2a are indeed functional inhibitory receptors. I also found that the CYT of IpLITR1.1b maintained its inhibitory potential in the presence of a DN-SHP-1, the implication being that IpLITR1.1b initiates inhibitory signals through a SHP-1-independent mechanism. In addition, the fact that inhibitory signaling initiated by IpLITR1.1b TR was not affected by the DN-SHP-1 reinforces that the SHP-1-independent recruitment is taking place in this CYT region, which is absent of canonical ITIMs but does contain tyrosine residues. The mechanisms of phosphatase-independent (i.e. SHP-1) inhibitory signaling pathways are poorly understood when compared to the more common ITIM-mediated recruitment and activation of SHP-1. However, there are some recent examples of SHP-1-independent mechanisms that play a role in inhibitory signaling events.

Work by Yusa et al. demonstrated that KIR2DL4 inhibits NK cytotoxicity through a SHP-1 and phosphotyrosine-independent mechanism [107]. KIR2DL4 encodes one ITIM and recruits SHP-2 but not SHP-1 as demonstrated by co-immunoprecipitation of SHP-2 with the KIR2DL4 CYT region [107]. Mutation of the tyrosine responsible for SHP-2 recruitment still did not inhibit the cytotoxicity response entirely. The inhibitory receptor LILRB1 has also been shown to recruit Csk in addition to SHP-1 [291]. Csk is a potent inhibitor of cellular signaling by its targeted phosphorylation of SFKs at a C-terminal regulatory tyrosine residue, which then induces conformational inactivation of these kinases [292]. The majority of information regarding Csk has focused on its role in T cell signaling where it negatively regulates the activity of stimulatory SFKs including Lck [293]. Csk has also been shown to regulate signaling pathways in drosophila suggesting that Csk has a highly conserved role in signaling in both vertebrate and invertebrate animals [294]. To date there have been no reports published demonstrating the involvement of Csk in immunoregulatory signaling in non-mammalian vertebrates. The only report on Csk function in teleosts presents a role for it regulation of the SFKs Fyn and Yes during zebrafish gastrulation [295].

In the previous chapters I demonstrated that the CYT region of IpLITR1.1b inhibits NK cells, and that this may be accomplished in a SHP-1-independent manner [296]. This suggests that additional inhibitory molecules are recruited to a region that does not normally recruit SHP-1 following phosphorylation. As described in Chapter V, KIR/IpLITR1.1b and KIR/ IpLITR1.1b TR inhibit NK cell-mediated cytotoxicity even in the presence of a catalytically inactive DN-SHP-1, while KIR2DL3 and KIR/IpLITR1.2a do not. Since the SHP-recruitment potential of the different KIR/IpLITR chimeras has

already been established in Chapter IV, in this Chapter I examined whether or not these KIR/IpLITR chimeras could also interact with an additional inhibitory signaling molecule.

My hypothesis was that if IpLITR1.1b-mediated inhibition is accomplished independent of SHP-1 or SHP-2 recruitment then there are alternative signaling molecule(s) that are recruited to this region that are accomplishing this inhibition of cytotoxicity. My objectives for this chapter are; i) to identify a candidate inhibitory signaling molecule recruited to the TM proximal CYT region of IpLITR1.1b; ii) to determine which tyrosine residue(s) is responsible for this recruitment; and iii) to develop protocols for the study of IpLITR-mediated signaling in channel catfish immune cells.

Herein, I report that the TM proximal CYT region of IpLITR1.1b recruits Csk through a non-ITIM phosphotyrosine-containing motif. The specific tyrosine residue responsible for recruitment of Csk was identified by site-directed mutagenesis. It is this same residue necessary for Csk recruitment that is also required for inhibitory signaling in NK cells. In the final portion of this chapter, I report on the use of biotinylated synthetic peptides corresponding to two different sequences encompassing tyrosine residues of IpLITR1.1b. One peptide corresponded to a canonical ITIM in the TM distal region of the CYT, encoded in both IpLITR1.1b and IpLITR1.2a, and the other peptide corresponded to the non-ITIM tyrosine sequence located in the TM proximal region of the CYT, which is responsible for recruitment of Csk and inhibition of cytotoxicity. By probing lysates of YTS cells, I confirmed that the phosphotyrosine residue in the proximal region does recruit Csk but not SHP-1 while the canonical ITIM peptide recruited SHP-1 as well as Csk. When I attempted to probe the channel catfish clonal B

cell line (3B11), I found that the canonical ITIM from the TM distal region recruited SHP-1 but not the ITIM-like peptide. The data in this chapter reveals that IpLITR1.1b inhibitory activity is mediated by a specific non-ITIM tyrosine that recruits Csk.

6.2 Results

6.2.1 Both the full-length CYT region and the TM proximal CYT region of IpLITR1.1b bind Csk

To test whether the CYT regions of IpLITR1.2a and 1.1b recruit Csk following tyrosine phosphorylation, I co-transfected the KIR/IpLITR chimeras with a plasmid encoding Csk into HEK 293T cells. Transfected cells were then treated with pervanadate, lysed, and immunoprecipitated with an anti-HA mAb. As shown in Fig. 6.1 (top panel), immunoprecipitation with anti-HA mAb followed by detection with an HRP-conjugated anti-HA mAb resulted in the detection of bands at the predicted size for each KIR/IpLITR chimera. When the HA-immunoprecipitated cell lysates were then probed with a pAb specific for Csk, no visible bands were detected in non-transfected cells (mock) or those transfected with Csk alone (Fig. 6.1; middle panel). However, HA-immunoprecipitated lysates from HEK 293T cells co-transfected with KIR/IpLITR1.1b or KIR/ IpLITR 1.1b TR and Csk demonstrated strong Csk immunoreactive bands (~40 kDa) when probed with the anti-Csk pAb (Fig. 6.1; middle panel). In comparison, the Csk plus KIR/IpLITR1.2a and Csk plus KIR/IpLITRΔCYT co-transfected cells recruited only faint Csk immunoreactive bands. The bottom panel of Fig. 6.1 shows the available Csk proteins in each of the cell lysates examined and demonstrates the difference between endogenous levels of Csk found in HEK 293T cells (mock) vs. the levels of Csk after transfection/ overexpression.

6.2.2. Y^{453} within the TM proximal CYT region of IpLITR1.1b is responsible for Csk recruitment

Following the demonstration of Csk recruitment to the TM proximal region of KIR/IpLITR1.1b (KIR/IpLITR1.1b TR), I wanted to identify which tyrosine residue of KIR/IpLITR1.1b TR was necessary for Csk recruitment. Outlined in Figure 6.2A is a schematic representation of the wild type (YY) and three mutated KIR/IpLITR1.1b TR constructs (FY, YF, and FF) that were generated for these experiments. The mutated tyrosine residues correspond to Y^{433} and Y^{453} of the native IpLITR1.1b sequence (ABI16050). Following transfections into HEK 293T cells, each receptor immunoprecipitated with the anti-HA mAb (Fig. 6.2B; top panel). When the IPs were probed with the Csk pAb, my results show that the wild type KIR/IpLITR1.1b TR YY construct recruited Csk (Fig. 6.2B; middle panel; 1.1b TR YY lane), similar to Figure 6.1. The KIR/IpLITR1.1b TR FY (Y^{433} to F^{433}) protein also co-immunoprecipitated Csk (Fig. 6.2B; middle panel). However, constructs containing the Y^{453} to F^{453} mutation (1.1b TR YF) or the Y^{433} to F^{433} plus Y^{453} to F^{453} mutations (1.1b TR FF) did not recruit Csk (Fig. 6.2B; middle panel). The bottom panel of Fig. 6.2B again shows the available Csk in each of the HEK 293T cell lysates.

6.2.3. Y^{453} within IpLITR1.1b TR is also responsible for inhibition

To examine if the differential Csk recruitment capacity observed for the different KIR/IpLITR1.1b TR proteins (i.e. 1.1b YY, FY, YF, and FF) also correlated with altered inhibitory function, I designed rVV encoding the various KIR/IpLITR1.1b TR tyrosine-to-phenylalanine mutants, infected NK cells and then performed killing assays similar to the NK cytotoxicity assays described in Chapter V. Surface expression of the receptors is

shown in Figure 6.3. The diminished ability of KIR/IpLITR1.1b TR YF and KIR/IpLITR1.1b FF to recruit Csk correlated precisely with their inability to abrogate the killing of 721.221-HLA-Cw3 targets at the various E:T ratios (Fig. 6.4). This is particularly evident when compared with the inhibitory activities of KIR/IpLITR1.1b TR YY and KIR/IpLITR1.1b TR FY that abrogated cell killing of .221-Cw3 target cells.

6.2.4. Cloning of zebrafish Csk

To demonstrate that Csk is present in fish, I cloned Csk from zebrafish embryos 96 hours post fertilization using primers specific for the zebrafish Csk sequence obtained from GenBank (accession number: NP_001071067.1). The resulting PCR reaction was loaded on a 1% agarose gel and was stained with ethidium bromide. I visualized a band corresponding to the predicted size of Csk at approximately 1500 base pairs (Fig. 6.5). When the sequence of Csk was compared with that of humans they shared 86% sequence identity. In fact, many of the signaling molecules known to be important in phosphatase-dependent and –independent inhibitory signaling are conserved between fish and humans (Table 6.1).

6.2.5. Synthetic phosphopeptides corresponding to IpLITR ITIMs demonstrate differential recruitment of SHP-1 and Csk

To further examine the recruiting potential of inhibitory IpLITR-types, I designed synthetic phosphopeptides corresponding to two separate CYT regions of IpLITR1.1b. One peptide was designed to correspond to the canonical ITIM encompassing Y^{475} found in the TM distal region of IpLITR1.1b, which is also shared with IpLITR1.2a (Fig. 6.6A). A second peptide was designed that corresponded to the non-canonical ITIM encompassing Y^{453}, which is responsible for Csk recruitment (Fig. 6.6A). Both of these

peptides were phosphorylated at the tyrosine residue and additional peptides were synthesized that were not phosphorylated to confirm that tyrosine phosphorylation was essential for recruitment of signaling molecules. YTS lysates were probed with the aforementioned synthetic peptides and then precipitated with streptavidin conjugated protein G beads. Proteins that were bound to the peptide-bead complex were eluted with reducing buffer, run on SDS-PAGE, and then SHP-1 and Csk were detected by WB. As presented in Figure 6.6B, the phosphopeptide corresponding to the ITIM encompassing Y^{475} recruited SHP-1 while the phosphopeptide corresponding to the region encompassing Y^{453} did not. Detection of the endogenous level of Csk is displayed as unprobed YTS lysate was also probed for Csk (Figure 6.6B, first lane). Lanes containing peptides that were not phosphorylated did not display any SHP-1 reactive bands. These same samples were also probed for the presence of Csk and both phosphopeptide sample lanes revealed recruitment of Csk as immunoreactive bands corresponding to the size of Csk were observed (Figure 6.6B). Again, non-phosphopeptides did not recruit any Csk (Figure 6.6B).

6.2.6. The synthetic IpLITR1.1b peptide containing Y^{475} recruits SHP-1 in the channel catfish 3B11 cell line

In order to detect whether the IpLITR synthetic peptides recruit teleost signaling proteins, the recruitment assays were repeated using the channel catfish 3B11 cell line as was performed previously with the YTS cell line. Samples were blotted with an anti-SHP-1 antibody and I detected a band corresponding to the size of SHP-1 (~70 kDa) in the sample lane containing the phosphopeptide encompassing Y^{475}, which is found in the context of a canonical ITIM. SHP-1 was not recruited to the phosphopeptide encompassing Y^{453} nor the non-phosphopeptides as no bands were detected in these lanes

(Fig. 6.7). Nitrocellulose blots of these samples were also probed with the anti-Csk pAb but no bands corresponding to the size of Csk were detected, even in the lysate lane (data not shown). To assess the total amount of protein recruited to the synthetic peptides, SDS-PAGE were stained with silver nitrate to assess total protein content, but this technique resulted in too much background and specific proteins were impossible to isolate (data not shown).

6.3. Discussion

In Chapter V I demonstrated that the TM proximal CYT region of IpLITR1.1b (tested as IpLITR1.1b TR) inhibited NK cell killing although this region did not recruit SHPs (Chapter IV) and was not affected by a recombinant DN-SHP-1. In this chapter, my goal was to further examine the possible mechanisms for this apparent SHP-1-independent inhibitory response. I found that following tyrosine phosphorylation, the kinase Csk was recruited by the CYT of KIR/IpLITR1.1b and KIR/IpLITR1.1b TR, but not KIR/IpLITR1.2a. This recruitment was dependent on an intact Y^{453} residue as shown by site-directed mutagenesis. Then by developing additional rVV encoding KIR/IpLITR1.1b TR with various tyrosine to phenylalanine mutations I discovered that the same tyrosine residue (Y^{453}) was also required for the inhibition of cytotoxicity towards target cells in the primary mouse NK cells. Furthermore, using NK cell killing assays, I determined that a Y^{433} to F^{433} did not have any functional effect. Therefore it appears that IpLITR1.1b Y^{453} is the major contributor to a SHP-independent inhibitory pathway involving Csk.

In mammals, Csk has been identified as a major endogenous inhibitor of SFK activity and thus is a key regulator of multiple kinase-dependent cellular responses [292,

297, 298]. Src-family protein tyrosine kinases are vital players in the control of cellular functions and aberrant regulation of SFK activity is associated with multiple diseases [299-302]. Aberrant control of SFK activities in immune cells also results in uncontrolled cellular proliferation (i.e. lymphoma) and immune-related pathologies (i.e. autoimmunity) [292, 303, 304]. The inhibitory actions of Csk are dependent on its ability to specifically phosphorylate a conserved tyrosine residue located at the C-terminus of SFKs (i.e. Lyn, Fyn, Hck, etc.), which inhibits their catalytic activities [292, 297, 298]. A non-catalytic homolog of Csk also exists, called CSK-homologous kinase (CHK), which also plays a pivotal role in the regulation of SFKs but it does so via non-covalent, allosteric protein–protein interactions with target kinases and not phosphorylation of the C-terminal regulatory tyrosine [297, 305].

Despite the knowledge that Csk is an important regulator of various cellular signaling events, recruitment of this regulatory kinase by immunoregulatory receptors and its ability to negatively influence cellular immune responses has only recently been recognized [291, 306]. This kinase-mediated inhibitory signaling in immune cells opposes the classical phosphatase-driven attenuation of immune cell responses but is likely as important in the regulation of immunity. Primarily located in the cytoplasm, Csk is known to be recruited to lipid rafts in the plasma membrane via an SH2 domain-mediated interaction with the Csk-binding protein (Cbp)/phosphoprotein associated with glycosphingolipid-enriched microdomains adaptor (PAG) [307, 308]. The recruitment of Csk to lipid rafts by Cbp/PAG promotes phosphorylation of Csk and places this regulatory kinase in close proximity to its targeted SFKs [297, 309]. Consequently, the Cbp/PAG-mediated membrane recruitment and activation of Csk has been implicated in

the negative regulation of immune cell signaling [310-312]. Recently, different ITIM-containing immunoregulatory receptor-types have also been demonstrated as Csk-binding proteins including LILR1, LAIR-1, and SIRPα [291, 306, 313]. Like the Csk-Cbp/PAG interactions described above, recruitment of Csk to these inhibitory membrane receptor-types places this protein at a location where it can effectively phosphorylate targeted SFKs rendering them inactive and incapable of initiating the proximal signaling events required for cellular activation such as the phosphorylation of ITAMs within stimulatory adaptor proteins. Additionally, since SFK-mediated phosphorylation of ITIMs is also necessary for phosphatase binding (i.e. SHP-1, SHP-2, and/or SHIP; [44, 79, 249]), concomitant or subsequent recruitment of Csk represents a potential auto-regulatory signaling mechanism by diminishing ITIM phosphorylation and abrogating phosphatase-mediated deactivation of intracellular signaling molecules like Vav1. This essentially would dictate how much phosphatase binding occurs during inhibitory receptor engagement and adding an additional level of immunoregulatory receptor control of cellular activation. Although Csk binding to LILR1, LAIR-1, and SIRPα appear to be ITIM-dependent events, there are several examples of other proteins that bind Csk in an ITIM-independent manner [314, 315], which now also includes IpLITR1.1b at Y^{453} within the non-ITIM 'AVYAQV' identified in this thesis. However, it is difficult to predict precisely how Csk interacts with all of its binding partners but a phosphorylated tyrosine is most certainly required [314].

While my functional data was dependent on the recruitment of a mammalian Csk to a teleost receptor, a Csk homolog is present in fish that shares >85% identity with the human protein (Table 6.1). In fact, many of the known inhibitory receptor signaling

mediators (e.g. SHP-1, SHP-2, SHIP, Csk, CHK, c-Abl, and Crk) and their specific targets (i.e. Vav, SLP-76, and several SFKs; reviewed in [44]) are also present in teleosts (Table 6.1), indicating that key mechanisms of immune cell inhibition are likely conserved between mammals and fish. Some of these signaling molecules have only recently been recognized as inhibitory mediators, which provide new insights into the mechanisms of immunoregulatory receptor signaling and new opportunities to explore these pathways in different vertebrates. Classically, ITIM-mediated signaling requires recruitment of intracellular phosphatases (e.g. SHP-1; [44, 50, 55, 79]) that dephosphorylate important proximal signaling proteins and metabolites (e.g. Vav1, SLP-76, IP_3, and $PI(3,4,5)P_3$) preventing the transmission of kinase-mediated signaling events and subsequent immune cell effector functions (i.e. cytotoxicity and cytokine secretion). However, it has also been reported that the ITIM-containing immune receptors, KIR and CD94/NKG2A induce a tyrosine phosphorylation-dependent inhibitory pathways via the actions of the non-receptor tyrosine kinase c-Abl and the Crk adaptor protein [316]. It is believed that following ligand engagement of KIR or CD94/NKG2A, SFK-mediated tyrosine phosphorylation of their ITIMs leads to an interaction between the c-Abl tyrosine kinase and the adaptor molecule Crk that culminates in the phosphorylation of Crk [316]. Phosphorylated Crk is then believed to contribute to cellular inhibition [316]. Interestingly, this mechanism is concurrent with SHP-1-mediated dephosphorylation of the guanine exchange factor Vav1 indicating that inhibitory immune receptor signaling is much more complicated than originally described and provides a new look into ITIM-mediated inhibitory signaling events. Teleost homologs for both c-Abl and Crk also exist

(Table 6.1) indicating that this novel inhibitory pathway may function in the regulation of fish immune cell responses.

Another inhibitory pathway mediated by immunoregulatory receptors is dependent on immune tyrosine-based switch motifs (ITSMs; [317]). While often encoded in CYTs that contain ITIMs, this motif binds SHP-1 and SHP-2 [318] but can also inactivate cellular signaling via a SH2-phosphatase-independent mechanism due to the selective recruitment of the adaptor proteins SH2 domain protein 1A (SH2D1A) and Ewing's sarcoma (EWS)-activated transcript 2 (EAT-2) [317, 319]. Like the other inhibitory signaling mediators described above (SHP-1, SHP-2, SHIP, Csk, CHK, c-Abl, and Crk), fish have SH2D1A and EAT-2 homologs (Table 6.1). Furthermore, the membrane distal region of IpLITR1.1b encodes an ITSM (TIYSQL) that may contribute to the ability of this receptor to influence leukocyte responses [278]. Clearly, more experiments are required to define the precise SHP-independent mechanisms of IpLITR-induced cellular inhibition.

In order to further characterize the signaling capability of specific IpLITR CYT motifs, I developed biotinylated synthetic peptides corresponding to a canonical ITIM encoded on both IpLITR1.1b and IpLITR1.2a as well as a unique motif found to recruit Csk from my previous experiments. Only the canonical ITIM peptide recruited endogenous SHP-1 in lysates from YTS cells while both the canonical ITIM and non-canonical motif recruited Csk. The SHP-1 recruitment data was consistent with my previous work that the CYT of IpLITR1.1b and IpLITR1.2a recruited teleost SHP-1

(Chapter III). These results support the conserved nature of SHP-1 recruitment to ITIMs in vertebrates.

Interestingly, the canonical ITIM phosphopeptide also recruited Csk even though my previous results in this chapter showed that only the non-ITIM containing TM proximal CYT region of IpLITR1.1b recruited Csk in transfected HEK293T cells. The reason for this discrepancy may be related to a similar observation with FcγRIIb in which agarose beads coated with phosphorylated peptides corresponding to the FcγRIIb ITIM bind both SHIP and SHP-1/2 *in vitro* while FcγRIIb selectively recruits SHIP *in vivo* [134, 320, 321]. When the amounts of phospho-ITIM peptide were lowered only SHIP and not SHP-1 was bound. This is because the affinity of the SH2 domain of SHIP is high enough to bind to phosopho-ITIM-coated beads but not the SH2 domain of SHP-1 [322]. Similarly in my experiments,the large amount of phospho-ITIM bound Csk *in vitro* whereas this was not observed *in vivo* with lower amounts of tyrosine phosphorylation associated with the transfected receptor. The understanding that phospho-ITIMs may lead to false positives for signaling molecule recruitment due to high concentration of phosphopeptides used must be kept in mind during these experiments and candidate molecule recruitment must be further tested by varying the concentration of phospho-ITIMs used in these peptide recruitment assays.

The synthetic peptides also proved useful for probing a channel catfish immune cell line (i.e. 3B11), for the identification of native teleost signaling molecules that may be utilized by IpLITRs *in vivo*. The synthetic peptide corresponding to the canonical ITIM recruited a band that was found to be the same size as the mammalian SHP-1 and

was detected on a Western blot with an antibody raised against the human SHP-1 (Figure 6.7). Consistent with the results with YTS lysates, the non-canonical ITIM peptide did not recruit SHP-1 in 3B11 lysates as well. Preliminary Western blotting of these samples with the anti-Csk pAb yielded multiple nonspecific bands although none were in the predicted size of Csk (data not shown). This was surprising because Csk is such a highly conserved molecular throughout vertebrates. One possible reason for this is that Csk may not be highly expressed in the 3B11 cell line. The 3B11 cell line is constantly proliferating and in this physiological level of constant growth and proliferation the amount of regulatory/inhibitory molecules may not be at the same level as cells undergoing normal limited growth and development. Further work is required in order to first determine which catfish cell lines do express Csk and then find a suitable cross-reactive anti-Csk antibody.

The results of the work in this chapter provide a unique look into the inhibitory signaling capacity of the inhibitory receptor IpLITR1.1b. I have demonstrated that both SHP-1-dependent and -independent pathways participate in the inhibitory functions of this protein, which can potently abrogate lymphocyte-mediated cellular cytotoxicity. The ability of an IpLITR CYT region to inhibit the effector function of mouse NK cells implies that evolutionarily conserved molecules facilitate inhibitory signaling in immune cells. This is reinforced by the identification of teleost homologs for a vast array of phosphatases, kinases, and adaptor molecules believed to participate in down-regulating immune cell responses (Table 6.1). Detailed studies are now required to: (i) confirm that Csk is a SHP-independent mechanism of IpLITR-mediated cellular inhibition; (ii) to determine if other SHP-independent inhibitory signaling pathways are engaged by

IpLITRs; (iii) to biochemically and functionally characterize inhibitory signaling molecules in teleost immune cells, and; (iv) to examine the impact(s) of IpLITR-mediated inhibitory signaling on fish immune defense against pathogens. The results presented in this chapter are an important first step in these directions.

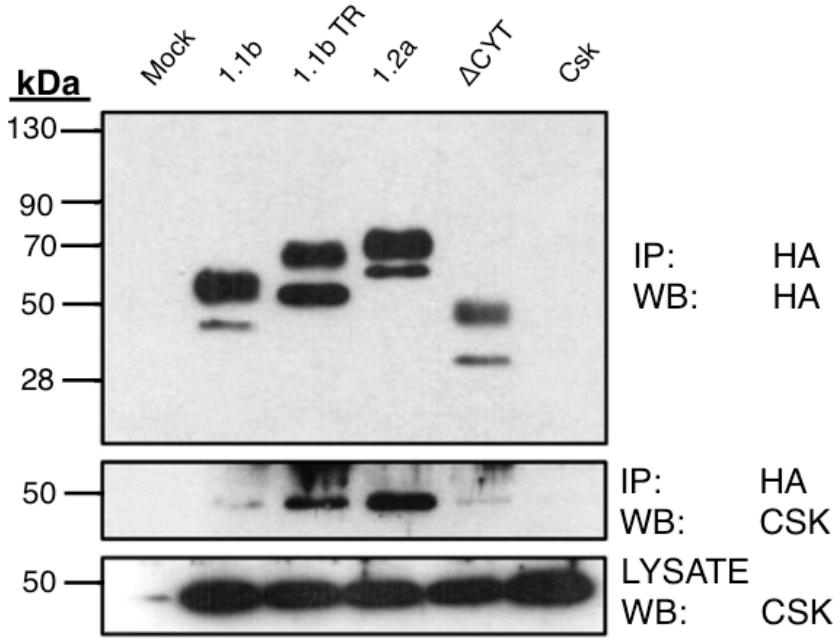

Figure 6.1. KIR/IpLITRCYT chimeras bind Csk. HEK 293T cells were transiently co-transfected with KIR/IpLITR1.2a, 1.1b, 1.1b TR, and ΔCYT and pXM113-Csk. After 48 hours the cells were treated for 15 min at 37 °C with pervanadate, lysed and then immunoprecipitated with anti- HA mAb. Samples were then separated on an 8% SDS–PAGE gel under reducing conditions, transferred to nitrocellulose and duplicate blots were probed with either an HRP conjugated anti-HA mAb (top panel) or with anti-Csk antisera followed by an HRP-goat anti-Rabbit IgG pAb (middle panel). Whole cell lysates were analyzed for total Csk expression (bottom panel). Mock lanes had lysates from non-transfected HEK 293T cells and Csk lanes are lysates from cells transfected with pXM113-Csk only. Data presented is a representative blot from two independent experiments.

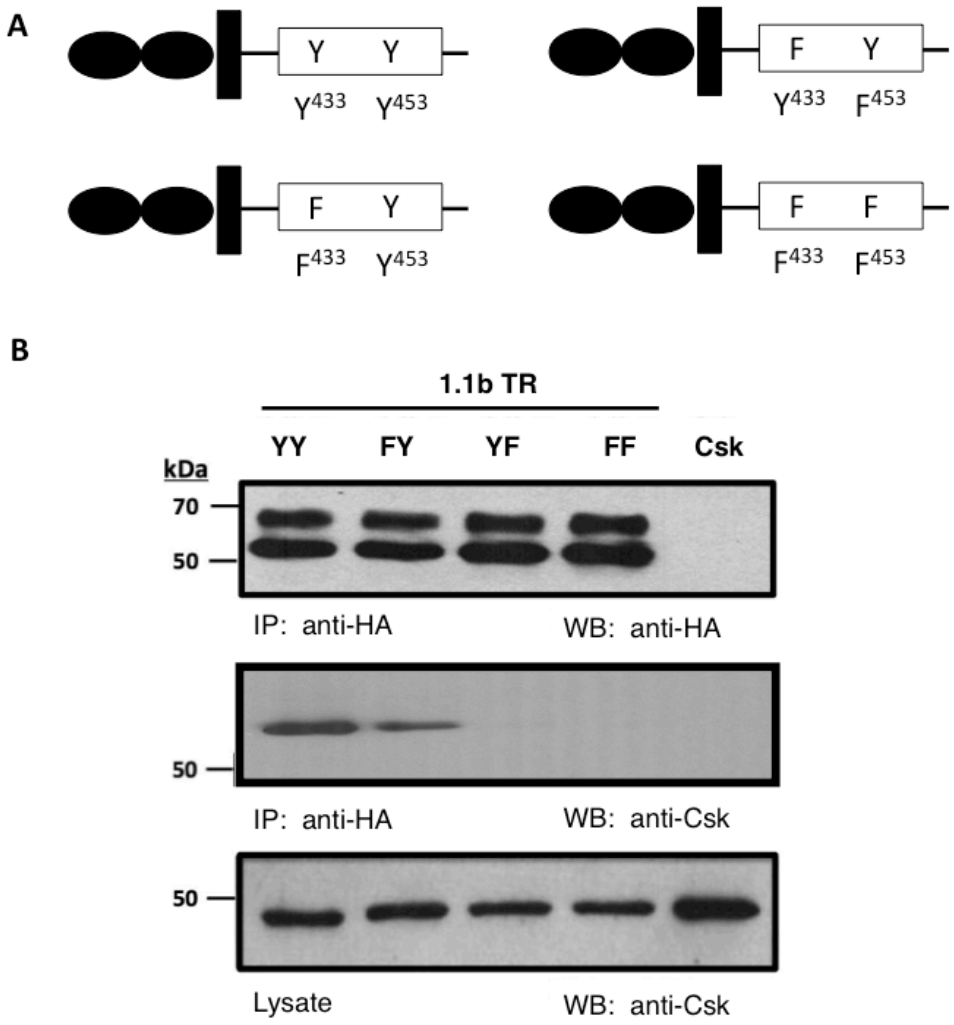

Figure 6.2 Tyrosine residue 453 within the TM proximal CYT region of IpLITR1.1b is responsible for Csk recruitment. A) Schematic representation of KIR/IpLITR1.1b TR tyrosine-to-phenylalanine mutants. B**)** HEK 293T cells were transiently co-transfected with wild-type KIR/LITR1.1b TR chimera or the 1.1b TR chimera with mutated tyrosines (i.e. 1.1b TR FY, YF, or FF) in conjunction with pXM113-Csk. After 48 hours, the cells were treated with pervanadate for 15 min at 37 °C, lysed and then immunoprecipitated with anti-HA mAb. Samples were then separated on an 8% SDS–PAGE gel under reducing conditions, transferred to nitrocellulose and duplicate blots were probed with either an HRP conjugated anti-HA mAb (top panel) or with anti-Csk antisera followed by an anti-Ig pAb conjugated to HRP (middle panel). Whole cell lysates were also analyzed for total Csk expression (bottom panel). Mock lanes were non-transfected HEK 293T cells and Csk lanes were cells transfected with pXM113-Csk only. Data presented is a representative blot from two independent experiments.

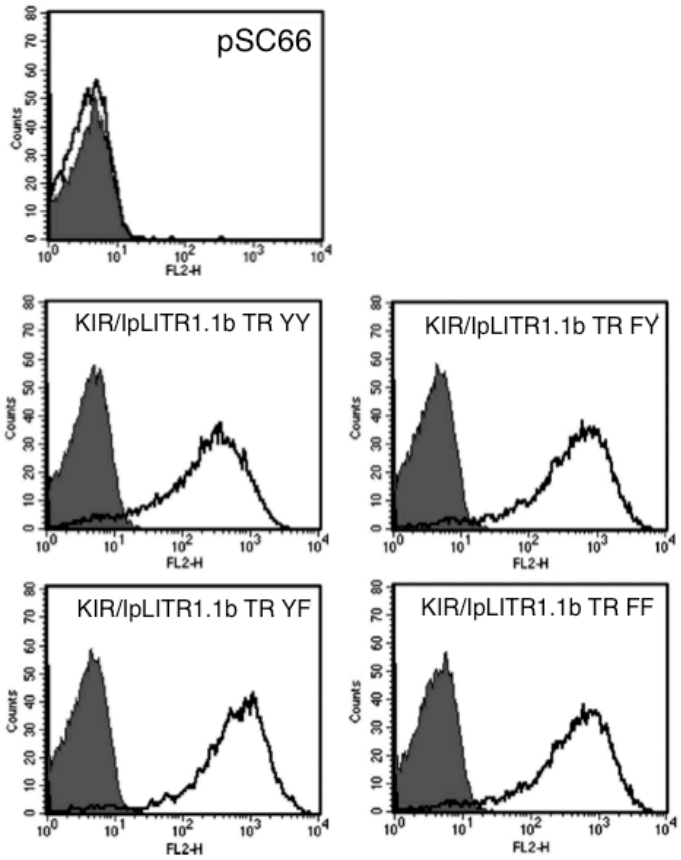

Figure. 6.3. Surface expression of KIR/IpLITR1.1b TR constructs in transfected NK cells. NK cells were infected with rVV-WR (MOI of 20) encoding an empty pSC66 vector, or the chimeric receptors KIR/IpLITR1.1b TR, KIR/IpLITR1.1b TR FY, KIR/IpLITR1.1b Tr YF, or KIR/IpLITR1.1b TR FF. The surface expression of each receptor was determined by flow cytometry using the anti-KIR mAb DX27 followed by staining with a PE-goat anti-mouse IgG pAb (empty histogram). The secondary pAb alone control is indicated by the solid histogram.

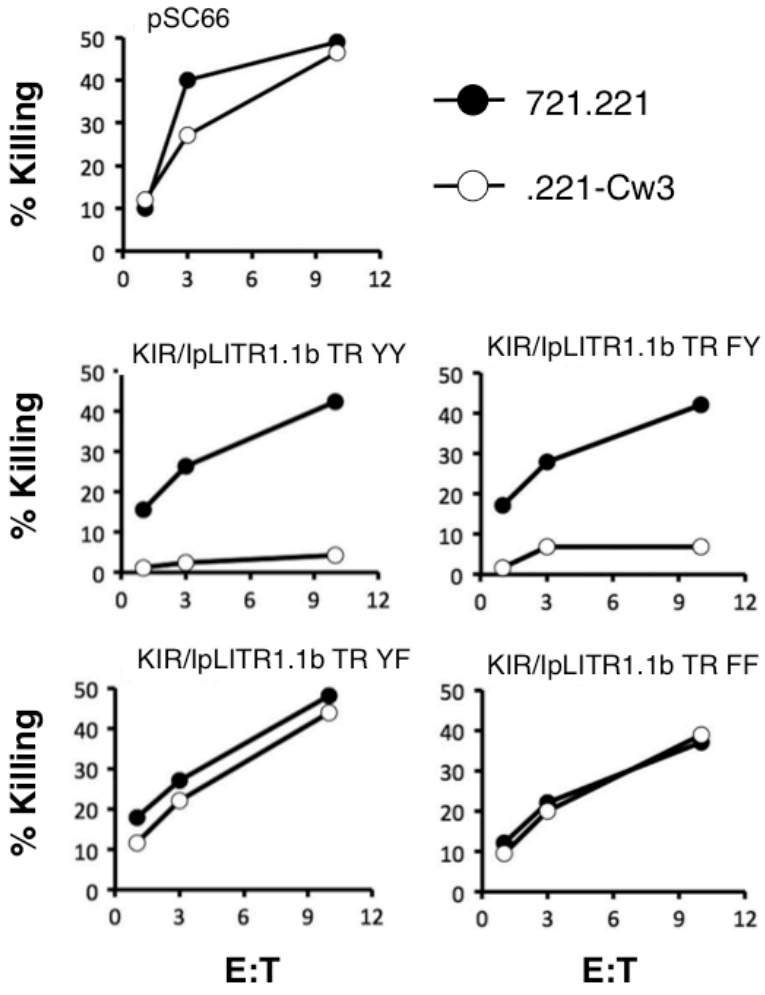

Figure 6.4. Tyrosine residue 453 within the TM proximal CYT region of IpLITR1.1b is responsible for inhibitory function. Mouse NK cells were infected for 4 h with rVV prior to mixing them with either 721.221 cells (black circles) or 721.221 cells expressing HLA-Cw3 (white circles) at E:T ratios of 1:1, 3:1, and 10:1. Effectors and targets were then incubated for 4 hours at 37 °C and target cell killing was measured from cellular supernatants using the ^{51}Cr release assay. ^{51}Cr release was calculated as: % lysis = 100 X (mean sample release - mean spontaneous release)/(mean total release - mean spontaneous release) and is represented as % killing on the y-axis of each graph. Data presented is a representative from two independent experiments.

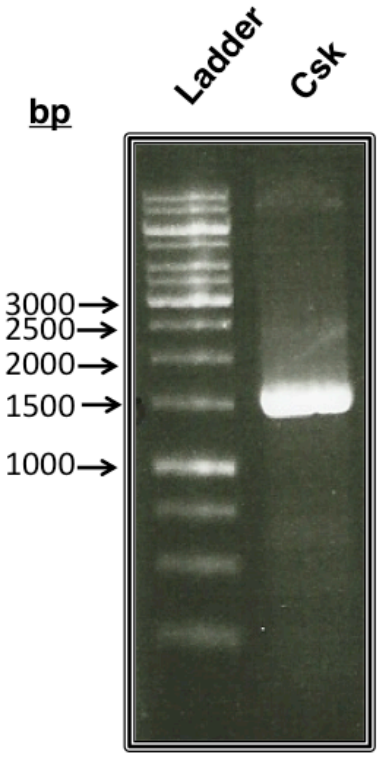

Figure 6.5. Cloning of teleost Csk. Csk was cloned and sequenced using primers generated from a cDNA sequence obtained from GenBank (Accession # NP_001071067.1). Zebrafish cDNA from embryos at 96 hours post fertilization were used as the cDNA source and the PCR product obtained is shown (~1500 bp).

Table 6.1. Phosphatases, kinases and other signaling molecules in mammals and fish[a].

Molecule[b]	Human[c]	Zebrafish[c]	% Identity[d]	Catfish[e]	% Identity[d]
SHP-1	AAA36610	CAZ68039	62	T67588	61
SHP-2	BAA02740	CAZ68072	90		
SHIP	AAB49680	XP_001923007	56	TC72632	45
SH2D1A	NP_001108409	XP_001920648	66	TC61594	64
EAT-2	AAM28522	NP_001189421	40	TC62921	42
Csk	NP_004374	NP_001071067	86	TC6373	87
CHK	NP_002369	XP_695792	63		
Vav1	AAH13361	XP_001119865	54	TC74369	55
c-Abl	NP_005148	XP_001337899	69		
Crk	NP_058431	NP_001003628	70	TC60454	69
Slp-76	EAW61478	NP_999882	38	TC63446	37
Cbp/PAG	NP_060910	XP_003200660	36	TC56935	27
c-Src	CAA26485	CAF06181	91	TC61748	91
Fyn	NP_002028	AAX47959	92	TC66318	73
Lck	AAH13200	NP_001035418	66	TC64454	68
Yes	NP_005424	NP_001013288	86		
Lyn	NP_002341	NP_001004543	76		
Fgr	NP_005239	NP_997946	71		
Blk	NP_001706	NP_001025391	69		

[a] Signaling molecules described throughout the manuscript that play important roles as mediators of immune cell inhibition and those that are targeted during immune cell inhibition are listed.

[b] Abbreviations defined throughout the manuscript.

[c] GenBank Accession numbers from http://www.ncbi.nlm.nih.gov/protein/.

[d] Percent amino acid identity when compared with the human sequence.

[e] TC (tentative consensus) Identifier as per DFCI (Dana Farber Cancer Institute, Harvard) Gene Index (http://compbio.dfci.harvard.edu/tgi/index.html) for channel catfish (*Ictalurus punctatus*).

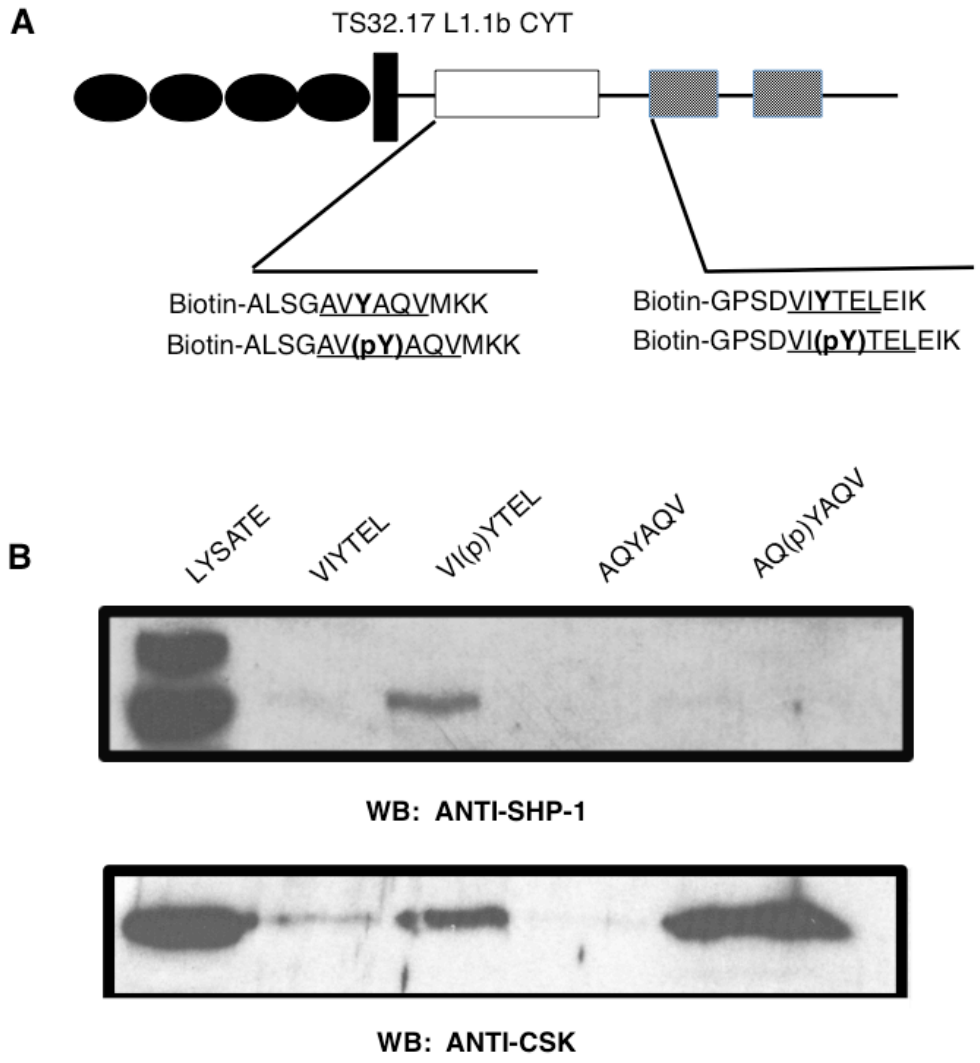

Figure 6.6. Synthetic peptides corresponding to separate tyrosine motifs in IpLITR1.1b have differential recruitment abilities for SHP-1 and Csk in YTS cell lysates. A) Schematic representation of biotinylated synthetic peptides corresponding to different regions of LITR1.1b. Peptides were synthesized in both phosphotyrosyl (indicated by pY) and non-phosphotyrosyl forms. The ITIM and ITIM like regions are underlined for each peptide. B) Synthetic IpLITR peptides (10µg) were added to YTS cell lysate ($1x10^6$ cells/mL) and incubated at 4^oC overnight. Streptavidin agarose beads were added to peptide-lysate mixture and incubated at 4^oC overnight. Agarose-peptide mixture was washed and resuspended in 2X SDS-PAGE reducing buffer and boiled for 10 minutes. Samples were loaded on an 8% SDS-PAGE then transferred to nitrocellulose membranes. Membranes were blotted with either an HRP conjugated anti-SHP-1 pAb or an anti-Csk pAb followed by an HRP conjugated goat anti-rabbit IgG pAb. Results are representative of three independent experiments.

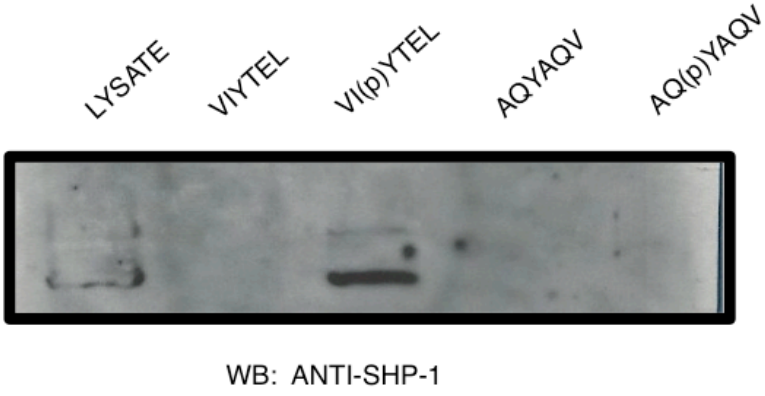

Figure 6.7. Synthetic peptides corresponding to canonical ITIM from LITR1.1b but not the non-canonical Csk binding motif recruits SHP-1 from the catfish 3B11 cell line. Synthetic IpLITR peptides (10µg) were added to 3B11 cell lysate ($1x10^6$ cells/mL) and incubated at 4^oC overnight. Streptavidin agarose beads were added to peptide-lysate mixture and incubated at 4^oC overnight. Agarose-peptide mixture was washed and resuspended in 2X SDS-PAGE reducing buffer and boiled for 10 minutes. Samples were loaded on an 8% SDS-PAGE then transferred to nitrocellulose membranes. Membranes were blotted with a HRP conjugated anti-SHP-1 pAb. Results are representative of three independent experiments.

CHAPTER VII

GENERAL DISCUSSION AND FUTURE DIRECTIONS

7.1. Summary of Findings

The discovery of the IpLITR family represents an important opportunity for further characterization of teleost immunoregulatory receptors. These immune proteins share key characteristics with other immunoregulatory receptors that promote stimulatory and/or inhibitory cellular signaling. In addition, based on their expression by multiple immune cell-types, I predicted that IpLITRs participate in the regulation of a variety of teleost immune cells (i.e. NK cells, B-cells, T-cells, and macrophages). Further insights into their roles in the regulation of teleost immunity requires functional studies aimed at characterizing their ligands, examining the signaling pathways engaged by various IpLITR-types, and understanding the roles of ligand binding and signaling on immune cell effector responses. Prior to the work presented in this thesis, there was no evidence to suggest that these receptors were, in fact, functional immune receptors capable of initiating or inhibiting leukocyte effector functions. During my tenure as a graduate student, the majority of studies in the laboratory focused on the characterization of stimulatory IpLITR-types while my research focused on examination of the inhibitory forms. The work of others in the lab demonstrated that stimulatory IpLITR-types associated with the teleost ITAM-containing adaptor molecules FcRγ and FcRγ-like (L) [156]. Ab cross-linking of a chimeric receptor consisting of the EC and TM of IpLITR and the CYT of FcRγ-L revealed that this adaptor molecule utilized conserved kinase

signaling pathways in order to induce phagocytosis and degranulation in the RBL-2H3 cell line [157].

At the same time, I focused my attention on the biochemical and functional characterization of inhibitory members of the IpLITR family (i.e. IpLITR members that contained at least one ITIM in their CYT region). My hypothesis was that IpLITR members that possess long CYT region with embedded ITIM motifs function as inhibitory immunoregulatory receptors in a similar fashion to ITIM-bearing IgSF receptors previously described in vertebrates such as the KIRs, LILRs, FcRs, and rodent PIRs [20]. Furthermore, I predicted that the ITIM-bearing IpLITR members, specifically IpLITR1.1b and IpLITR1.2a, are phosphorylated at tyrosine residues within the context of the ITIM and that this tyrosine phosphorylation provides a docking site for inhibitory signaling molecules, such as the phosphatases SHP-1 and SHP-2 [323]. Subsequently, IpLITR-mediated recruitment of intracellular phosphatases would result in the abrogation of an immune cell response such as NK cell-mediated killing of target cells. Recruitment and activation of SHP-1 and SHP-2 is a common strategy for the regulation of immune responses and prevention of autoimmune disorders. The specific research aims of my thesis were (1) To biochemically establish the inhibitory signaling potentials of two putative inhibitory IpLITRs termed IpLITR1.1b and IpLITR1.2a; and (2) To functionally establish the inhibitory role of IpLITR1.1b and IpLITR1.2a in mouse splenic NK cells.

I began to address my research questions by first developing chimeric expression constructs, which consisted of the EC and TM of KIR2DL3 fused to the CYT of either IpLITR1.2a, IpLITR1.1b, a truncated form of IpLITR1.1bTR, and an expression construct with the CYT deleted save for 8 aa, which contained no signaling motifs

(Chapter III). These receptors were expressed on the surface of HEK 293T cells and determined to be phosphorylated at tyrosine residues following pervanadate treatment as indicated by Western blotting with a phosphotyrosine antibody (4G10). Following tyrosine phosphorylation, IpLITR1.1b and IpLITR1.2a recruited both zebrafish SHP-1 and SHP-2 (Chapter III). Recruitment of SHPs is a hallmark of functional inhibitory receptors and this supported the hypothesis that at the biochemical level these ITIM-containing IpLITRs are bona fide inhibitory receptors. In these studies I did not explore the possibility that SHIP was also recruited to these receptors as it has been demonstrated that a conserved leucine at the Y+2 position is necessary for recruitment to FcγRIIb and this aa pattern is not present in the ITIM and ITIM-like regions of IpLITR1.1b and IpLITR1.2a [320, 324]. However, it has been demonstrated that SHIP recruitment to FcγRIIb in human cells is also dependent on the co-recruitment of growth factor receptor-bound protein 2 (Grb2) or Grb2 associated protein (GAP) [325, 326]. Grb2 is an adaptor molecule that can act as a linker between two separate molecules as well as strengthen a preexisting weak interaction between two molecules (reviewed in [327]). IpLITR1.1b also contains a Grb2 recruitment motif (YxN) and so the recruitment of Grb2 or GAP may also be a potential mechanism for the increased recruitment capability of IpLITR1.1b CYT region for a diverse array of CYT signaling molecules. However, further studies would be required to unequivocally establish whether or not SHIP and other signaling molecules are recruited to these IpLITRs.

The recruitment of SHP-1 and SHP-2 was confirmation that IpLITR1.1b and IpLITR1.2a have inhibitory potential but the next step was to determine that these receptors were capable of regulating an immune cell effector response. To address this I

chose to examine NK cell killing of target cells, which is a tightly regulated event in other animals [77]. Importantly, this regulation is significantly dependent on immunoregulatory receptor-mediated recruitment of intracellular phosphatases that effectively abrogate the NK cell killing machinery [79]. I transfected and expressed the chimeric expression constructs on the surface of the NK cells isolated from the spleens of mice through infection with rVV. IpLITR1.1b and IpLITR1.2a both inhibited NK cell-mediated cytotoxicity of target cells expressing the ligand for KIR2DL3 (.221-Cw3) at a level similar to the WT KIR2DL3 (Chapter V). Cytotoxicity assays with the KIR/IpLITR1.1b TR chimera also demonstrated efficient inhibition of cytotoxicity. This result was surprising as the CYT region does not contain any canonical ITIMs and the previous work in Chapter IV showed that, although the CYT region of IpLITR1.1b recruited SHP-1 and SHP-2, the CYT of IpLITR1.1b TR did not recruit these inhibitory phosphatases. Then using a rVV encoding a DN-SHP-1 I co-infected the mouse NK cells with the chimeric KIR/IpLITR expression constructs to investigate the functional role of SHP-1 recruitment in IpLITR-mediated inhibition of NK cell killing. In the presence of the DN-SHP-1, both KIR2DL3 and KIR/IpLITR1.2a reverted back to near normal levels of cytotoxicity while there was no change in the levels of inhibition of cytotoxicity with KIR/IpLITR1.1b and KIR/IpLITR1.1b TR. This result indicated that there was a motif in the TM proximal CYT region contained in KIR/IpLITR1.1b TR that was capable of initiating inhibitory signaling in a manner that did not require SHP-1. At this point in my research, I had information that addressed my first objective: IpLITR1.1b and IpLITR1.2a were true functional inhibitory receptors that both had the potential to recruit SHP-1 and SHP-2, most likely with canonical ITIMs serving as docking sites. In

addition to this, I discovered that the TM proximal CYT region of IpLITR1.1b also initiated inhibitory signals in a SHP-independent manner. The remainder of my thesis work then focused on investigating alternative mechanisms for how this non-ITIM and non-SHP-1 mechanism of inhibitory signaling may be occurring.

My next approach was to attempt to identify candidate molecules that could be involved in the inhibitory signaling mediated by the unique CYT region of KIR/IpLITR1.1b TR. This required me to broaden my search from looking solely into signaling molecules that have been reported to bind ITIMs (e.g. SHP-1 and SHP-2) and look more generally at phosphotyrosine binding molecules, namely molecules that contain SH2 domains. The SH2 domain was originally identified as a noncatalytic region of approximately 100 aa conserved among cytoplasmic tyrosine kinases (CTKs) and later included cytoplasmic tyrosine phosphatases (CTPs) [328]. This domain recognizes a phosphorylated tyrosine residue embedded within the context of a short amino acid motif (4-6 aa) [329]. This motif will vary in terms of amino acid substitutions depending on the kinase or phosphatase family in question but the general pattern is pYxxI/V/L. It is important to note that this motif can also be defined as a partial ITIM so it is not unusual for a region containing an ITIMs to also have the ability to recruit Csk as well. The concept of the ITIM was first formed when Daeron et al. reported that FcγRIIb, a low-affinity IgG receptor, inhibits cellular effector function in B cells through a phosphorylated tyrosine signaling motif [330]. The ITIM in FcγRIIb recruited SHIP, which dephosphorylates its target PLC-γ, resulting in the inhibition of B-cell effector functions [320]. Around the same time, it was found that the CYT region of KIR2DL3 recruited SHP-1, which contained two SH2 domains [331]. Currently, there are

significantly more reports for recruitment of SHP-1 as the hallmark of an inhibitory receptor including a recent study on the recruitment of SHP-1 to a TCR-like molecule in the sea lamprey, a jawless vertebrate [279]. Less common are reports of SHIP recruitment to inhibitory receptors, the few examples being FCRL3 on B-cells [332], the killer cell lectin-like receptor G1 (KLRG1) expressed on NK cells and a small subset of T-cells [333], and the mast cell receptor leukocyte mono-Ig-like receptor 1 (LMIR1) [334].

I searched for candidate inhibitory molecules that contained SH2 domains and have also been reported to bind to phosphotyrosine motifs in inhibitory immunoregulatory receptors. From these searches I hypothesized that the inhibitory kinase Csk could potentially bind to the phosphotyrosine motifs in the CYT. This prediction was based largely on the previous work by Songyang et al., who identified a Csk-binding consensus sequence of Y(T/A/S)(K/R/Q/N)(M/I/V/R) using a peptide library [315]. This Csk-binging motif fit within the aa region containing Y^{453}, in the sequence AVYAQV, of the IpLITR1.1b CYT region. Therefore, I examined recruitment of Csk to this motif in HEK 293T cells by co-transfecting them with the various KIR/IpLITR constructs and a human Csk construct. Co-immunoprecipitation of the chimeric receptor and blotting with an anti-Csk pAb revealed that the TM proximal CYT region of IpLITR1.1b does indeed bind Csk. Site-directed mutagenesis then confirmed that Y^{453} of the motif AVYAQV was in fact the tyrosine residue responsible for recruitment of Csk. To support that this residue is necessary for the SHP-1-independent inhibitory signaling, the site-directed mutants of KIR/IpLITR1.1b TR were also expressed in mouse NK cells and, consistent with my Csk recruitment results, Y^{453} was confirmed to be required for

inhibitory signaling. To my knowledge, this is the first work demonstrating that Csk is recruited to a non-ITIM motif in an inhibitory immunoregulatory receptor that does not also have the potential to recruit SHP-1.

To finish my work, I focused on designing reagents that would provide a greater insight into teleost inhibitory signaling pathways by directly investigating the channel catfish immune model system and moving away from previous work in mammalian models. I designed synthetic peptides corresponding to different tyrosine-containing regions from the CYT region of IpLITR1.1b. One peptide was the aa sequence surrounding Y^{453}, which recruited Csk and the other corresponded to the canonical ITIM found in both IpLITR1.1b and IpLITR1.2a, DIYTEL. These peptides were biotinylated to allow for their precipitation from cell lysates for the identification of endogenous proteins they may recruit. This technique provides a means to determine the molecules that are recruited to various CYT regions of IpLITRs without the need to express the full-length receptor on the cell surface. From YTS lysates I found that while both the canonical and non-canonical ITIM regions recruited SHP-1 only the ITIM-like peptide recruited Csk, as determined by WB analysis (Chapter VI). The SHP-1 recruitment was similar in the channel catfish B-cell line, 3B11, where only the canonical ITIM recruited the phosphatase but not the ITIM-like region. A band corresponding to the size of Csk was not detected in the WBs of the 3B11 lysates but this may be due to the pAb not successfully cross-reacting to the teleost Csk or there being very low levels of endogenous Csk that make it difficult to detect.

To summarize the findings from this thesis, it is now evident that IpLITR1.1b and IpLITR1.2a are bona fide inhibitory immunoregulatory receptors. Functionally, both

receptors can inhibit cytotoxicity responses in primary mouse NK cells and biochemically they both recruit SHP-1 and SHP-2. In addition, I also discovered that IpLITR1.1b inhibits cytotoxicity through a non-ITIM phosphotyrosine motif and demonstrated that the inhibitory kinase, Csk, recruits to this specific region. There have been very few reports on Csk recruitment by inhibitory receptors with LILRB1 [291] and LAIR-1 [306] being two examples. Most work has focused on its interplay with CD45, a stimulatory phosphatase, in the regulation of TCR signaling (reviewed in [293]). My findings are unique in that the TM proximal CYT region of IpLITR1.1b, which is responsible for the recruitment of Csk, does not also facilitate the recruitment of SHP-1 or SHP-2, which is the case for both LILRB1 and LAIR1 [291, 306]. The TM proximal CYT region of IpLITR1.1b, which contains the Csk-recruitment motif, is a splice variant of IpLITR1.2a, which is lacking that region and only contains a canonical ITIM and ITSM. Taken at face value, it may appear from my results that these fish receptors have a separate CYT region responsible for inhibitory signaling through Csk recruitment and another for SHP-1 and SHP-2 recruitment while the examples of Csk in mammals have been for Csk recruitment at motifs that also recruit SHP-1 and/or SHP-2. One possible reason for this dichotomy is that the longer CYT tail may have evolved this unique role in inhibitory immunoregulatory signaling from an original role that was much different. One possibility is that ancient IpLITRs and their ability to recruit Csk may have been utilized during embryogenesis and tissue development where it has been shown that Csk plays a necessary role in proper gastrulation [295]. These receptor signaling motifs may have then been adapted later on for a role in immune signaling where the TM proximal CYT region in IpLITR1.1b, which recruits Csk, is sufficient for inhibition but not

necessary as the distal CYT region, also found in IpLITR1.2a, is capable of inhibitory signaling as well. The further examination of these receptors in the context of channel catfish leukocyte signaling will reveal

Another perspective on these findings of SHP-independent inhibitory signaling is that what I have presented in terms of Csk recruitment is not unique to IpLITRs or teleosts but rather it is just not as commonly identified at this point in time. As the definition of an inhibitory signaling motif is broadened from the current ITIM to a more inclusive definition that takes into account inhibitory regulatory enzymes other than SHP-1 and SHP-2, more receptors will be found to have the ability to recruit molecules with inhibitory roles including, but not restricted, to Csk. One recent example is a report from Peterson *et al*. that demonstrates that inhibition of NK effector responses is also established through the phosphorylation of Crk [316]. This phosphorylation leads to the inactivation of Crk and its dissociation with other singaling molecules involved in the NK effector response.

In summary, my findings bring additional support to the notion that the strategy of SHP-1 and SHP-2 recruitment to ITIMs of immunoregulatory IgSF receptors is conserved in vertebrates. Also this work provides novel evidence that reinforces that immunoregulatory IpLITR signaling is complex and while it does share some conserved features with prototypical immunoregulatory inhibitory receptors it may also reveal unique and unexpected features as well. A schematic diagram is provided to describe the findings of this thesis in terms of IpLITR inhibitory signaling as well as addressing some of the unknowns that still remain (Fig. 7.1).

7.2. Future Directions

7.2.1. Further characterization of the role of Csk in IpLITR1.1b-mediated inhibitory signaling.

From the results of this thesis it is clear that IpLITR1.1b recruits a mammalian Csk at a specific region surrounding Y^{453}. To further confirm that Csk is responsible for the inhibitory signaling, I would repeat the cytotoxicity assays using the KIR/IpLITR1.1b TR Y to F mutants expressed in primary splenic NK cells derived from an inducible $Csk^{-/-}$ mouse, as $Csk^{-/-}$ phenotype leads to death in utero [335]. If inhibition is still observed at a similar level then the results would suggest that although Csk is recruited to this region it may not be necessary for inhibiting NK cytotoxicity.

Another approach to confirm that Csk is a true candidate for IpLITR inhibitory signaling would be to repeat the KIR/IpLITR1.1b TR cytotoxicity assays but after combining the effector cells with the targets to then perform intracellular flow cytometry and stain the cells with antibodies specific for the phosphorylated form of the regulatory tyrosine region of the substrates Fyn or Lck [292]. If the cells are stained positive with these antibodies, it would provide further evidence that Csk recruitment to the IpLITR CYT plays a dominant role in cellular inhibition.

To reinforce that IpLITR1.1b recruits Csk in teleost, Csk would be cloned from zebrafish or channel catfish cDNA, similar to what was accomplished in Chapter IV with zebrafish SHP-1 and SHP-2, and then ligated into an epitope-tagged expression vector. This expression construct would be co-transfected into HEK 293T cells, the cells would be pervanadate-stimulated, lysed, then immunoprecipitated with an antibody specific for the epitope-tagged IpLITR. This experiment would be very similar to what was

accomplished to determine IpLITR recruitment of both SHP-1 and SHP-2 [278]. Since Csk is highly conserved among vertebrates, I predict that IpLITR1.1b does indeed recruit teleost Csk. The results of this experiment would serve as further confirmation that the recruitment of Csk by IpLITR1.1b is a strategy that is utilized by the channel catfish to mediate immunoregulatory signaling.

At this point in time I have determined which aa region of IpLITR1.1b recruits Csk, but it is still unclear what residues are essential for this recruitment apart from the phosphotyrosine residue. In a similar technique that was used to characterize the SHIP-binding site in FcγRIIb [320, 336], I would exchange residues between the canonical IpLITR ITIM used in the phosphopeptide studies with that of the Csk-recruiting phosphopeptide.

7.2.2 Examination of alternative leukocyte effector responses influenced by IpLITR1.1b and IpLITR1.2a signaling

The effector response that I examined in this thesis was NK cytotoxicity. In addition to this response, IgSF immunoregulatory receptors play many diverse roles in cellular function including adhesion [140], proliferation [337], cytokine and chemokine release [116], phagocytosis [12, 338], migration [339, 340], and survival [341]. IpLITRs are a large family of IgSF receptors that are expressed in leukocytes of both lymphoid and myeloid cell lineage including T-cells, B-cells, NK-like cells, and macrophage and monocytes [2, 3]. By examining if and how these inhibitory IpLITRs regulate other effector responses in various leukocyte types we can gain a greater appreciation for the potential roles of IpLITR and IgSF immunoregulatory receptors in general. At the same time, by examining different effector functions it may also provide novel information on

what signaling molecules are recruited to IpLITRs and, conversely, the studies of IpLITRs could provide novel information towards what signaling molecules are involved in these immunoregulatory networks that are conserved in vertebrates.

7.2.3. Examining the role of the ITSM in IpLITR1.1b signaling

There is one ITSM found surrounding the TM distal CYT tyrosine residue in both IpLITR1.1b and IpLITR1.2a. The ITSM is defined by the motif TxYxxV/I/L and was first described by Shlapatska et al. as a CYT motif on CD150, also termed signaling lymphocytic activation molecule (SLAM) [342]. CD150 contains a long CYT with two ITSMs and is capable of transmitting either positive or negative signals through the differential recruitment of SH2D1A, also termed SLAM associated protein (SAP), or SHP-2, respectively [342, 343]. Not only does SH2D1A competitively block the recruitment of SHP-2 but it also recruits the SFK Fyn to transmit activation signals in T-cells leading to the release of IL-4 [344, 345]. In Chapter VI, I identified SH2D1A in teleosts, which is highly homologous to that SH2D1A in mammals. To address whether SH2D1A is recruited to the ITSM in IpLITR1.1b and IpLITR1.2a, this molecule would also have to be cloned from channel catfish cDNA and then ligated into an epitope-tagged expression vector. The epitope-tagged SH2D1A would then be co-transfected with the various KIR/IpLITR expression constructs and co-immunoprecipitaion assays performed. Western blotting would then be used to determine if SH2D1A is recruited to the IpLITR CYT and which region specifically would be determined using site-directed mutagenesis.

It would also be interesting to further explore whether additional molecules are recruited to the ITIM-like region in the proximal area of IpLITR1.1b. To address this I

would immunoblot the samples of peptide pull-downs performed from YTS lysates (Chapter VI) in an attempt to identify any other signaling molecules that are recruited. In addition, I would also perform silver stains on the samples to visualize all of the proteins that are recruited to this motif. From that point I would isolate discrete bands and attempt to identify them through mass spectrometry. My preliminary work using this assay has not yet yielded promising results as it has been difficult to visualize discrete bands due to high levels of background staining on the gel. This approach requires further optimization that may involve more stringent washing condition to wash away less specific binding partners.

It is also possible that SHIP may be recruited to this motif in a manner similar to that of FcγRIIb [134]. To date, the specific AxYAQV motif has not been identified in the CYT of vertebrate inhibitory receptors outside of IpLITRs. For example the alanine at the Y-2 position was shown to have no negative effect on the binding potential of the ITIM in FcγRIIb to SHIP [134] but it was shown to ablate any recruitment of SHP-1 to KIR2DL3 [51]. This molecule may have a more permissive SH2 domain, which allows it to bind to additional hydrophobic residues at the Y-2 position such as an alanine.

7.2.3. Identification of IpLITR ligands

One of the most intriguing and difficult questions that remain about IpLITRs is "to what do they bind?" Through sequence alignments and comparative homology modeling it was revealed that the IpLITR EC domains D1 and D2 have similar predicted three-dimensional properties with the corresponding domains of the LILR family [3]. Furthermore, conservation of key MHC-I-binding residues was located at similar positions within the membrane distal region of D1 between representative IpLITRs and

group 1 LILRs [3]. These results suggest that LITRs may function as MHC-I binding receptors [3]. It must be stated that this work is hypothetical and true MHC-I binding by any IpLITR members has not been established. Furthermore, many IgSF receptors that were previously thought to recognize one specific ligand have now been shown to engage something entirely different making it difficult to accurately predict the types of ligands that are recognized. Some examples include; FCRLs, which were once predicted to bind antibodies have now been shown to bind MHC-II (for FCRL6) [234] and the poxvirus immunoevasin protein (for FCRL5) [235]; CHIRAB1 in chicken has been found to be a high affinity IgY Fc receptor even though it has more features in common with the LILR family, which recognize MHC-I [10] and/or the viral MHC-I homolog, UL18 [346], than the FcR family [132]; Siglec-5 recognizes the heavily glycosylated clotting factor VIII and Von Willebrand factor [347]; CD150 (SLAM) on T-cells and B-cells engages another CD150 on the surface of APCs and function as an adhesion molecule but is also a receptor for the measles virus [14, 348]; and it has also been recently reported that the CD300 family of receptors, along with the TREM receptors and the teleost DICP family, recognize the lipids phosphatidylserine and phosphatidylethanolamine [11, 184, 338]. These are just a few examples that reinforce that the possible ligand for an IgSF receptor is incredibly diverse and one must be aware of this when approaching this task.

In order to identify the ligand(s) for IpLITRs I would employ a broad approach so as to optimize the most opportunities to find the candidates. One approach would be to develop Fc-fusion proteins that consist of the EC domains of the IpLITR fused to the Fc region of a human or mouse IgG isotype [349, 350]. Once these chimeras are developed they can be screened against an array of cells and protein extracts from both microbial

and eukaryotic cellular sources by Far western techniques. In addition, lipid components can be plated in cell culture plates and then screened with the Fc-fusion proteins. Some components that could be tested would include various lipids and carbohydrates as seen to bind the CD300 family of receptors and the Siglecs, respectively [216, 347, 351].

An alternative approach used by Carlyle *et al*., to identify that the self-molecule osteoclast inhibitory ligand (ocil) was the cognate ligands for the inhibitory natural killer receptor (NKR)-P1B/D one in which a rapid functional assay as well as a ligand-binding staining reagent were developed [352]. The functional assay utilized the BWZ reporter cell line, which produces β-galactosidase in response to stimulation [353]. This cell line would be transfected with a cDNA containing the EC domains of various stimulatory and inhibitory forms of IpLITRs fused to the TM and CYT of a modified CD3ζ subunit. These reporter cells would be combined with various cell lines from the channel catfish including B-cells [166, 354], T-cells [252, 355], monocytes/macrophages [255], and NK-like cells [170, 253, 356] and examined to see whether these cells may trigger the release of β-galactosidase from the reporter cells indicating that the presence of something on the surface of the cells that is recognized by the IpLITR EC domains. Alternatively, tetramers consisting of the soluble form of the IpLITR EC domains would be used as staining agents to detect potential ligands on the surface of cells that reacted to the BWZ reporter cells. If a cell line is found that does indicate a potential ligand is expressed then a cDNA library could be constructed from this cell line and the cDNAs expressed in an easily transfectable cell line such as HEK293T cells. These cells could be screened with the IpLITR tetramers and when a cell is found to be positively stained then the transfected cDNA would be sequenced to identify the potential ligand. The discovery of

the IpLITR ligand(s) will add to a greater understanding of this receptor family and its relationship to other immunoregulatory receptors families in vertebrates.

7.3. Summary

Many comparative immunology studies are still at the point where a great deal of focus is spent identifying genes and gene families that may play a role in the immune system, while less attention has been spent on functionally characterizing these recently identified immune genes. I started my thesis research with a recently identified family of receptor genes that displayed the characteristics of either stimulatory or inhibitory immunoregulatory receptors. In this thesis I reported on the biochemical and functional characterization of the CYT regions of two prototypical inhibitory IpLITRs, IpLITR1.2a and IpLITR1.1b. From these findings I have revealed that IpLITR1.1b and IpLITR1.2a are bona fide functional inhibitory immunoregulatory receptors and that the potency of the immune response is on par with the prototypical mammalian inhibitory receptors, specifically KIR2DL3. I have also presented that the method of inhibition mediated by these receptors is through both SHP-1 dependent and independent mechanism. This is the first report of evidence for the mechanism of inhibitory receptor signaling in teleost and in addition, it is one of the few reports in vertebrates that demonstrate a phosphatase independent mechanism of inhibitory immunoregulatory signaling. I believe that these findings have significantly added to the current knowledge about inhibitory receptor signaling in teleosts. Furthermore, the research contributions described in this thesis provide a greater understanding into the complexity of inhibitory receptor signaling in vertebrates.

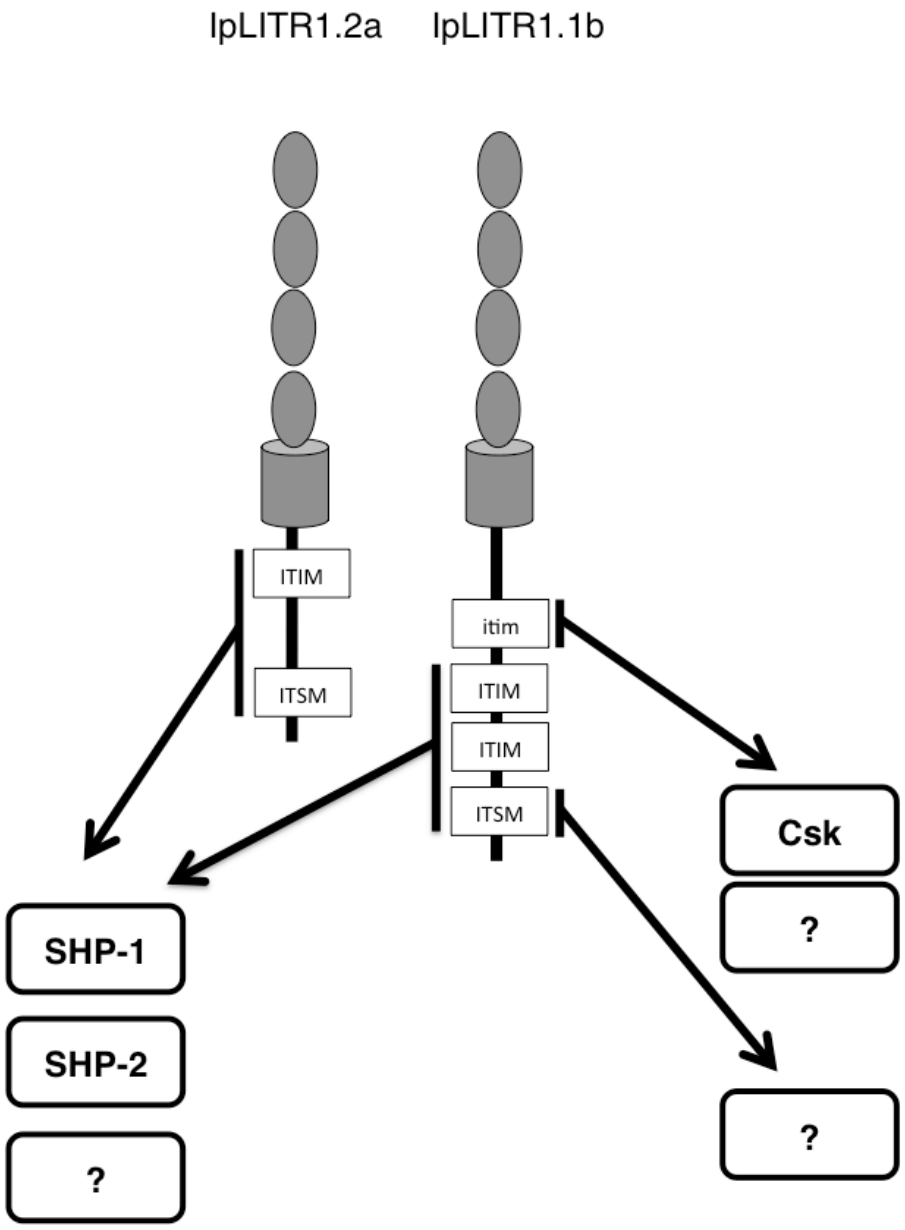

Figure 7.1. Schematic representation of the findings in this thesis. The arrows point to the various signaling molecules that are recruited to the region encompassed by the vertical line. Rounded boxes with question marks represent the possibility for additional signaling molecules to be involved but their identities are unknown at this time.

CHAPTER VIII

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