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Channel catfish leukocyte immune-type receptor mediated inhibition of cellular cytotoxicity is facilitated by SHP-1-dependent and -independent mechanisms

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ABSTRACT

Channel catfish (*Ictalurus punctatus*) leukocyte immune-type receptors (IpLITRs) are immunoregulatory proteins belonging to the immunoglobulin superfamily that likely play an important role in the regulation of teleost immune cell effector responses. IpLITRs are expressed by myeloid and lymphoid subsets and based on their structural features can be classified as either putative stimulatory or inhibitory forms. We have recently demonstrated at the biochemical and functional levels that stimulatory IpLITR-types induced intracellular signaling cascades resulting in immune cell activation. Alternatively, we have shown that putative inhibitory IpLITRs may abrogate immune cell responses by recruiting teleost Src homology 2 (SH2) domain-containing cytoplasmic phosphatases (SHP) to their tyrosine-containing cytoplasmic tails. In the present study, we used vaccinia virus to express recombinant chimeric proteins encoding the extracellular and transmembrane regions of human KIR2DL3 fused with the cytoplasmic tails of two putative inhibitory IpLITRs (i.e. IpLITR1.2a and IpLITR1.1b) in mouse spleen-derived cytotoxic lymphocytes. This approach allowed us to study the specific effects of IpLITR-induced signaling on lymphocyte killing of B cell targets (e.g. 721.221 cells) using a standard chromium release assay. Our results suggest that both IpLITR1.2a and IpLITR1.1b are potent inhibitors of lymphocyte-mediated cellular cytotoxicity. Furthermore, using a catalytically inactive SHP-1 mutant in combination with site-directed mutagenesis and co-immunoprecipitations, we also demonstrate that the IpLITR1.2a-mediated functional inhibitory response is SHP-1-dependent. Alternatively, IpLITR1.1b-mediated inhibition of cellular cytotoxicity is facilitated by both SHP-1-dependent and independent mechanisms, possibly involving the C-terminal Src kinase (Csk). The involvement of this inhibitory kinase requires binding to a tyrosine residue encoded in the unique membrane proximal cytoplasmic tail region of IpLITR1.1b. Overall, this represents the first functional information for inhibitory IpLITR-types and reveals that catfish LITRs engage SHP-dependent and -independent inhibitory signaling pathways to abrogate lymphocyte-mediated killing.

1. Introduction

In response to infection a complicated series of receptor-mediated signaling events are necessary for initiation, propagation, and subsequent termination of potent immune cell effector responses. These cellular immune responses (e.g. phagocytosis, degranulation, cytokine secretion, and cytotoxicity) are vital for immune defense against pathogens and are in part controlled by

Abbreviations: IpLITRs, *Ictalurus punctatus* leukocyte immune-type receptors; SH2, Src homology 2; SHP, Src homology 2 domain-containing cytoplasmic phosphatase; Csk, C-terminal Src kinase; ITAM, immune receptor tyrosine-based activation motif; SFK, Src-family protein tyrosine kinases; SYK, spleen tyrosine kinase; PI3-K, phosphatidylinositol 3-kinases; IgSF, immunoglobulin superfamily; FcRs, Fc receptors; KIRs, killer cell immunoglobulin-like receptors; LILRs, leukocyte immunoglobulin-like receptors; NKRP1, natural killer receptor P1; ITIMs, immune receptor tyrosine-based inhibition motifs; CYT, cytoplasmic tail; SHIP, SH2-domain containing inositol 5-phosphatase; NK, natural killer; rVV, recombinant vaccinia virus; LAK, lymphokine activated killer; Y, tyrosine; IL-2, interleukin 2; MHC I, major histocompatibility class I; HLA, human leukocyte antigen; mAb, monoclonal antibody; pAb, polyclonal antibody; HA, hemagglutinin; PE, phycoerythrin; HRP, Horseradish peroxidase; tr, truncated; F, phenylalanine; WR, Western Reserve; DN, dominant-negative; TK-, thymidine kinase-deficient; MOI, multiplicity of infection; D-PBS, Dulbecco's phosphate buffered saline; Cr, chromium; E:T, effector to target; PEI, polyethylenimine; IP, immunoprecipitation; ED, extracellular domains; TM, transmembrane; kDa, kiloDalton; CHK, CSK-homologous kinase; Cbp/PAG, Csk-binding protein/phosphoprotein associated with glycosphingolipid-enriched microdomains adaptor; LAIR, leukocyte-associated Ig-like receptor-1; SIRP, signal-regulatory protein; SH2D1A, SH2 domain protein 1A; EAT-2, Ewing's Sarcoma-activated transcript 2.

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subsets of co-expressed stimulatory and inhibitory immunoregulatory receptors that are coupled with distinct intracellular signaling modules (Barrow and Trowsdale, 2008; Lanier, 2008; Long, 1999; Takai, 2005). In general, stimulatory immunoregulatory receptor-types activate immune cells by recruiting adaptor proteins encoding immune receptor tyrosine-based activation motifs (ITAMs). Following receptor engagement with specific ligands, ITAMs are phosphorylated by Src-family protein tyrosine kinases (SFKs), which then interact with downstream mediators such as spleen tyrosine kinase (SYK) and/or phosphatidylinositol 3-kinases (PI3Ks) that in turn potentiate a series of cellular activation cascades (Bakker et al., 2000; Kuster et al., 1990; Gergely et al., 1999; Lanier, 2008; Lanier et al., 1998; Underhill and Goodridge, 2007; Van den Herik-Oudijk et al., 1995; Wu et al., 2000). Many of these stimulatory receptors are germline-encoded and belong to either the immunoglobulin superfamily (IgSF) (Barclay, 2003) or the C-type lectin superfamily (Weis et al., 1998). Examples of immunoregulatory proteins with stimulatory functions include Fc receptors (FcRs; Falk and Ravetch, 2006; Nimmerjahn and Ravetch, 2007, 2011; Takai, 2005), killer cell Ig-like receptors (KIRs; Parham, 2004; Stanietsky and Mandelboim, 2010; Vilches and Parham, 2002), leukocyte Ig-like receptors (LILRs; Brown et al., 2004; Katz, 2006; Thomas et al., 2010), triggering receptors expressed on myeloid cells (Ford and McVicar, 2009), natural cytotoxicity receptors (e.g. NKp30, NKp44, and NKp46; Biassoni et al., 2001; Yokoyama and Plougastel, 2003), CD94/NKG2 (Gunturi et al., 2004; López-Botet et al., 1997), NK receptor-P1 (NKRP1; Carlyle et al., 2004), and Ly49 receptors (Anderson et al., 2001). In addition to these stimulatory forms, inhibitory receptor-types also play an indispensible role in the regulation of cellular immune responses (Long 2008, Long et al. 1997 and Steevels and Meyaard 2011).

Inhibitory receptors establish the activation threshold of immune cells and attenuate stimulatory receptor-induced effector functions. Like their stimulatory counterparts, these receptors are also present within the FcR, KIR, LILR, CD94/NKG2, NKRP1, and Ly49 families. When engaged by specific ligands, inhibitory receptors recruit cellular phosphatases, which play an important role in down-regulating immune cell responses (Katz, 2006; Long, 1999; Ravetch and Lanier, 2000; Vivier and Daeron, 1997). The inhibitory capacity of these receptors is primarily dependent on the presence of immune receptor tyrosine-based inhibition motifs (ITIMs) within their cytoplasmic tail (CYT) regions (Beebe et al., 2000; Burshtyn et al., 1996, 1997, 1999). Ligand-induced phosphorylation of the tyrosine residue embedded within ITIMs (S/I/V/LxYxxI/V/L; an x indicates any amino acid) leads to the recruitment of SH2 domain-containing cytoplasmic phosphatases (SHP-1, SHP-2, and SH2-domain containing inositol 5-phosphatase; SHIP), which dephosphorylate various intracellular activation signaling intermediates (Burshtyn et al., 1997, 1999; Imhof et al., 2006). Recent studies have indicated substrate specificity for inhibitory phosphatases suggesting that they are not random or non-specific inhibitors of SFK-induced phosphorylation events. For example, when recruited to ITIMs, SHP-1 exhibits specificity for the guanine nucleotide exchange factor Vav1 resulting in its dephosphorylation and inability to activate Rac1 (Stebbins et al., 2003). Since Vav1 plays an important role in T cell activation events including synapse formation and receptor clustering (Tybulewicz, 2005), SHP-1-mediated dephosphorylation of Vav1 can likely block a range of cellular immune responses including natural killer (NK) cell cytotoxicity (Long, 2008). There are also SHP-independent inhibitory signaling mechanisms that occur in immune cells. For example, Csk binds to phosphorylated tyrosines present in the CYT region of ligand-engaged immunoregulatory receptors (Sayos et al., 2004; Veillette et al., 1998; Verbrugge et al., 2006). Csk is a potent inhibitor of cellular signaling by its targeted phosphorylation of SFKs at a C-terminal tyrosine residue, which then induces conformational inactivation of these kinases (Okada et al., 1991). Overall, complex mechanisms of receptor-induced inhibitory signaling pathways are required to abrogate potent cellular immune effector responses (Long, 2008; Ravetch and Lanier, 2000; Vivier et al., 2004).

Although much less is known about the specific immune receptor-types and signaling events involved in controlling cellular immunity in non-mammalian vertebrates, several immunoregulatory receptor families have been discovered in avian, amphibian, and teleost species (Yoder and Litman, 2011). Often, these immune proteins share key structural features with mammalian immunoregulatory proteins known to control and coordinate leukocyte responses (Yoder and Litman, 2011; Montgomery et al., 2011). Detailed sequence analyses, examination of phylogenetic relationships, and several functional studies have provided important information required for interspecies comparisons of vertebrate immunoregulatory receptor networks and their predicted phylogenetic origins (Yoder and Litman, 2011). One example from teleosts are the channel catfish LITR proteins, which represent a large and polymorphic immune receptor family with signaling potential predicted to augment or abrogate catfish immune cell responses (Montgomery et al., 2011; Stafford et al., 2006). Although IpLITR ligands remain unknown, recent biochemical and functional studies have revealed that associations with ITAM-encoding adaptor molecules as well as ITIM-mediated recruitment of cellular phosphatases are key requirements for IpLITR-mediated stimulatory and inhibitory functions, respectively (Mewes et al., 2009; Montgomery et al., 2009). We have also demonstrated that IpLITR-adaptor associations induce ITAM-dependent intracellular signaling and functional responses such as degranulation and phagocytosis in transfected immune cells, confirming the stimulatory nature of certain IpLITR-types (Cortes et al., 2012).

Expanding on our previous findings that putative inhibitory IpLITRs recruited SHP-1 and SHP-2 in an ITIM-dependent fashion (Montgomery et al., 2009), the focus of the present study was to examine the inhibitory capabilities of two different ITIM-containing IpLITR-types by addressing the following questions: (i) do the ITIM-bearing CYT regions of IpLITR1.2a (ABI16051) and IpLITR1.1b (ABI16050) 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? Herein we show that these receptors function as potent inhibitors of lymphocyte-mediated cellular cytotoxicity (i.e. target cell killing). Specifically, we used recombinant vaccinia virus (rVV) to express the previously reported 'inhibitory' KIR/IpLITR$_{CYT}$ constructs (Montgomery et al., 2009) in mouse lymphokine activated killer (LAK) cells. This allowed us to determine the inhibitory effects of ITIM-encoding IpLITR CYT regions on cellular cytotoxic responses. Co-expression of the KIR-LITR$_{CYT}$ with a catalytically inactive SHP-1 recombinant protein revealed that IpLITR-mediated inhibition of the LAK cell killing response was only in part a SHP-1-dependent mechanism. The inhibitory functions of KIR-LITR$_{CYT}$ 1.1b, which encodes the CYT region of IpLITR 1.1b (ABI16050), was not affected by the inactive SHP-1 mutant, indicating an unexpected SHP-1-independent mechanism of immune cell inhibition. Subsequently, site-directed mutagenesis and co-immunoprecipitations revealed that Csk is possibly an additional player in IpLITR 1.1b-mediated abrogation of the LAK cell killing response. 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.