Published in final edited form as:
Immunol Rev. 2008 August ; 224: 70–84. doi:10.1111/j.1600-065X.2008.00660.x.

Negative signalling by inhibitory receptors: the NK cell paradigm

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Summary

Receptors carrying immunoreceptor tyrosine-based inhibition motifs (ITIM) in their cytoplasmic tail control a vast array of cellular responses, ranging from autoimmunity, allergy, phagocytosis of red blood cells, graft versus host disease, to even neuronal plasticity in the brain. The inhibitory function of many receptors has been deduced on the basis of cytoplasmic ITIM sequences. Tight regulation of natural killer (NK) cell cytotoxicity and cytokine production by inhibitory receptors specific for MHC class I molecules has served as a model system to study the negative signalling pathway triggered by an ITIM-containing receptor in the physiological context of NK–target cell interactions. Advances in our understanding of the molecular details of inhibitory signalling in NK cells have provided a conceptual framework to address how ITIM-mediated regulation controls cellular reactivity in diverse cell types.

Keywords

inhibition; innate immunity; natural killer cell; signalling; tyrosine phosphatase

Introduction

This review covers the regulation of cellular activation by cell surface receptors that carry immunoreceptor tyrosine-based inhibition motifs (ITIM) in their cytoplasmic tail, with an emphasis on the mechanism of inhibition. The sequence of the ITIM was defined as V/I/LxYxxL/V by sequence alignment, phosphorylated peptide binding specificity, and functional tests (1–4). Since then, ITIM-containing receptors (ITIM-R) have grown into a large family with expression in many different cell types, and inhibitory functions for the control of various cell activation pathways. This type of negative regulation may be an ancestral system, as many ITIM-Rs are expressed in bony fish (5). In general, inhibition exerted by ITIM-Rs is only local and transient. It does not induce a cell-wide or sustained non-responsiveness, but abrogates activation signals when and where they occur. NK cells are well suited as a model system for biochemical and functional analysis of the inhibitory signals delivered by ITIM-R, which allow NK cells to kill infected cells but spare healthy ones. NK cells express several ITIM-Rs, for which the ligands on target cells are well defined. NK cells selectively kill target cells that have lost expression of MHC class I. Early observation of this phenomenon led to the “missing self” hypothesis (6). Target cell killing by NK cells is not occurring by default in the absence of inhibition, but requires signalling by activation receptors upon recognition of their specific ligands on target cells. An inhibitory receptor specific for MHC class I was first identified in mice, as Ly49A (7). Similar negative regulation operates in human NK cells, which express several inhibitory receptors specific for different HLA molecules. These include NKG2/CD94

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with specificity for HLA-E and killer-cell immunoglobulin-like receptors (KIR) that are specific for allotypes of HLA-C and HLA-B (8,9).

The primary questions about the role of ITIM-Rs were: What are the ligands of ITIM-Rs? How is the inhibitory signal transmitted? What specific functions are regulated in vivo, and how? While genetic approaches using mouse knockouts have been very informative in this respect, for many of the receptors within the ITIM family the negative signalling pathways that control specific cellular functions have not been delineated. Progress in unravelling the inhibitory mechanism that operates in NK cells has provided some insight into how ITIM-Rs function.

ITIM: Early history

The ITIM was initially defined as a tyrosine-containing sequence in the cytoplasmic tail of FcγRIIb, which caused inhibition of B-cell activation and influx of extracellular Ca^{2+} when FcγRIIb is co-engaged with the BCR (10,11). The sequence was further mapped to within 13 amino acids that include tyrosine 309 (12). It was identified as ITIM, by analogy with the immunoreceptor tyrosine-based activation motif (ITAM), which is found in several signalling adaptor proteins associated with receptors such as the TCR, BCR, and Fc receptors. The tyrosine-phosphorylated cytoplasmic tail of FcγRIIb binds to protein tyrosine phosphatase SHP-1 (13) and to the inositol phosphatase SHIP (14). Although phosphorylated FcγRIIb binds to both phosphatases, only SHIP is required for inhibition. SHIP dephosphorylates PI-3,4,5-P_3 into PI-4,5-P_2, thereby preventing membrane-association of Tec-family tyrosine kinases and sustained activation of PLC-γ (15,16).

Functional characterization of an inhibitory KIR revealed binding of SHP-1 to cytoplasmic phospho-tyrosines and led to the definition of the ITIM sequence as V/I/LxYxxL/V (1). The requirement for a specific amino acid at position −2, relative to the tyrosine, was unusual. Sequences of peptides bound to the isolated SH2 domains of tyrosine phosphatases SHP-1 and SHP-2 from large peptide libraries were in agreement with the ITIM sequence (4,17). SHP-1 and SHP-2 bind to the ITIMs of both KIR and mouse Ly49 inhibitory receptors (18). Selective binding of the SHIP SH2 domain to the FcγRIIb ITIM, and of SHP-1/2 to the KIR ITIM, is determined by a leucine at position +2 in FcγRIIb, and a leucine/isoleucine at position −2 in the KIR ITIM (19,20). Whereas FcγRIIb requires SHIP but not SHP-1 for inhibition, KIR inhibition is mediated by SHP-1 and not SHIP. Co-ligation of BCR and FcγRIIb in the chicken DT40 B cell line resulted in inhibition in the absence of SHP-1, but not SHIP (21). Conversely, co-ligation of BCR and a chimeric FcγRIIb carrying a KIR cytoplasmic tail resulted in inhibition in the absence of SHIP but not SHP-1. Furthermore, the reverse chimeras were expressed in NK cells and inhibition of lysis by the HLA class I ligand of KIR on target cells was tested (22). Inhibition by the KIR cytoplasmic tail was prevented by a dominant-negative form of SHP-1, but not SHIP. Conversely, a dominant-negative form of SHIP, but not SHP-1, prevented inhibition by the KIR/FcγRIIb chimeric receptor expressing the FcγRIIb cytoplasmic tail. Therefore, SHIP is dispensable for inhibition by KIR, while SHP-1 is dispensable for inhibition by FcγRIIb.

Amino acid substitutions in the two ITIMs present in the cytoplasmic tail of KIR provided functional evidence for the role of both ITIMs in transmitting inhibitory signals (3,23). The first, N-terminal and membrane-proximal, ITIM is not only essential, but also sufficient for inhibition. Phosphorylation of the first ITIM alone results in the preferential binding to SHP-2 (24). The first ITIM of receptors NKG2A (25) and Siglec-7/9 (26) are also dominant in their inhibitory function. However, due to the opposite transmembrane orientation of lectin-like type II proteins, the first, N-terminal, ITIM in NKG2A is membrane-distal. The lectin-like inhibitory Ly49 receptors carry a single ITIM. It is likely that assembly of Ly49 into a homodimer provides two ITIMs for optimal inhibitory function.

Recruitment of the tyrosine phosphatases SHP-1 and SHP-2 through binding of their tandem SH2 domains releases the catalytic domain from an inhibitory, intramolecular interaction with the N-terminal SH2 domain (27). Thus, recruitment and activation of tyrosine phosphatases by phosphorylated ITIMs provided a potentially simple explanation for inhibition. By dephosphorylation of tyrosines in activation receptors (such as ITAM-associated receptors, DAP10-associated receptor NKG2D, and the tyrosine-bearing receptor 2B4) and various tyrosine–dependent signalling components of the activation pathway, SHP would simply shut down any phospho-tyrosine-dependent signal (Fig. 1). However, this prevailing model for inhibition by KIR via SHP dephosphorylation of phosphotyrosine-dependent activation signals demanded experimental validation. Is ITIM-bound SHP well suited for rapid dephosphorylation of a large number of phosphotyrosines, which are distributed among multiple receptors and other proteins? Perhaps inhibition relies on a more sophisticated and reliable mechanism, which is designed to bring together active SHP and specific proteins that control activation signals.

The negative signalling pathway in NK cells

Early studies with NK cells, which were mixed with target cells that were either sensitive or resistant to lysis, showed that inhibition occurs at a very early signalling step, even prior to release of intracellular Ca^{2+} (28). The formation of PLC-γ–LAT complexes is also inhibited by the binding of inhibitory KIR to HLA class I in NK cells (29). The coalescence of lipid rafts at the interface with target cells is also inhibited by KIR engagement (30). Studies of the inhibitory mechanism have also been conducted with T cells. Engagement of inhibitory receptor CD94/NKG2A on T cells by MHC class I on tumor cells inhibited cytotoxicity, TNF-α release, and tyrosine phosphorylation of Lck and ZAP70, but not the TCR ζ chain (31). A $KIR2DL1^+$ tumor-specific T cell clone in contact with tumor cells that expressed an HLA-C ligand failed to phosphorylate ZAP70, LAT, and Vav, did not polarize lipid rafts, and failed to accumulate F-actin towards the tumor cell (32). Consistent with a block that is upstream of actin cytoskeleton rearrangements, engagement of an inhibitory KIR by MHC class I ligand on target cells greatly reduced the recruitment of actin and myosin IIA to the WIP–WASp complex (33). Myosin II is required for the degranulation but not granule polarization towards target cells (34). HLA-E attached to beads was sufficient to induce CD94/NKG2A accumulation, tyrosine phosphorylation, and SHP-1 recruitment to the zone of NK cell–bead contact, while causing exclusion of F-actin and ERM proteins (35). Several questions arise: How do ITIM-Rs accumulate at the inhibitory synapse and how is their phosphorylation achieved? How does inhibitory KIR prevent proximal activation signals, before Ca^{2+} flux and actin cytoskeleton–dependent processes? Could it be due to dephosphorylation of a specific substrate by ITIM–bound SHP?

The rapid accumulation of KIR at inhibitory immune synapses, through binding to HLA class I on target cells, has unusual properties. Accumulation occurs even in the absence of actin polymerization and ATP production (36). On the other hand, KIR accumulation is enhanced by Zn^{2+} (36), which is also required for optimal inhibitory function (37). Binding to HLA class I ligands alone is sufficient to induce receptor clustering, as KIR2DL1 accumulated at the site of contact with insect cells expressing HLA-Cw4 (38), and CD94/NKG2A clustered towards HLA-E coupled to beads (35). Accumulated KIR at inhibitory NK cell immune synapses segregates from LFA-1 into distinct regions (36,39). The distribution pattern varies according to the density of the HLA-C ligand on target cells: Segregation of HLA-C from ICAM-1 into a central cluster on target cells occurred only at high HLA-C density (40).

Rapid phosphorylation of ligand-bound ITIM-Rs is an obvious prerequisite for inhibitory function. Lck is a candidate Src-family kinase for the phosphorylation of ITIMs (41–44). However, it is still unclear how phosphorylation is achieved. Two, non-exclusive, hypotheses

have been proposed. One is that phosphorylation of ITIMs by a Src-family kinase occurs in *trans* during co-engagement of inhibitory and activating receptors at the inhibitory NK cell immune synapse. However, the highly efficient inhibition of proximal signals by KIR engagement is not easily reconciled with an ITIM phosphorylation that is itself dependent on the very signals ITIMs have to stop. The other hypothesis is that clustering of inhibitory receptors is sufficient to recruit a Src-family tyrosine kinase, which phosphorylates the ITIMs. However, association of a kinase with ITIMs has not been detected, except for the binding of Lck to the mouse inhibitory NKR-P1B receptor through a CxCP motif in the cytoplasmic tail (44). ITIM phosphorylation without detectable association with a kinase is obviously possible, and would explain how CD94/NKG2A recruits SHP-1 upon binding to HLA-E on beads (35). The strong tyrosine phosphorylation of ITIM-Rs obtained by the treatment of cells with the tyrosine phosphatase inhibitor pervanadate implies that ITIMs are constitutively phosphorylated, and that their basal unphosphorylated state is maintained by tyrosine phosphatase activity.

High-resolution imaging of KIR by detection of fluorescence resonance energy transfer (FRET) between KIR and an anti-phosphotyrosine antibody revealed that phosphorylated KIR forms a few small clusters within the NK–target cell contact area, whereas total KIR is distributed over a larger area (45). This result raises interesting questions. Are clusters of phospho-KIR–SHP-1 complexes scanning the contact area in search of activation signals, or do they co-localize with clusters of activation receptor signalling? As TCR signalling occurs in small clusters within immune synapses, which move from a peripheral region towards the center of the synapse (46), it is possible that activation signals in NK cell immune synapses are also delivered by discrete signalling complexes, and that phosphorylated KIR is specifically targeted to such signalling receptor clusters.

Identification of substrates targeted for dephosphorylation by ITIM-bound SHP-1 could provide new information about critical signals for NK cell activation, and about the mechanism of inhibition. As SHP-1 is active only when tethered through its SH2 domains to phospho-ITIMs, a generalized, global dephosphorylation of many different substrates (Fig. 1) seemed unlikely. A substrate selection restricted in space and time would more likely focus on a few proteins. Identification of a substrate during inhibition of NK cells by KIR binding to an HLA class I ligand on target cells was performed using a “functional substrate trapping” approach. A KIR2DL1–SHP-1 chimera was generated, which had a full-length SHP-1 inserted in place of the cytoplasmic ITIMs (Fig. 2). An Asp to Ala substitution (DA) in the catalytic domain of SHP-1 results in a catalytically inactive phosphatase, which can still bind phosphotyrosine substrates. The only detectable substrate trapped during KIR2DL1–SHP-1(DA) binding to cognate HLA-C was Vav1 (47). The essential role of Vav1 in T cell immunological synapse formation and receptor clustering (48), and the tyrosine phosphorylation–dependent Rac1 activation by the guanine exchange factor domain of Vav1, suggests that Vav1 dephosphorylation by SHP-1 may be sufficient to block NK cell cytotoxicity.

Phosphorylation of Vav1 would have to occur independently of actin polymerization, if Vav1 were to be a key target for dephosphorylation by SHP-1 during ITIM–dependent inhibition. Consistent with this prediction, trapping of Vav1 occurred in the presence of cytochalasin D (47). Furthermore, actin-independent phosphorylation of Vav1 is induced by binding of the β_2 integrin LFA-1 to insect cells transfected with the ligand ICAM-1 alone (49). Inhibition of LFA-1–dependent lysis of insect cells expressing ICAM-1 and HLA-C by binding of inhibitory KIR to peptide-loaded HLA-C was reported, implying that LFA-1 signalling is sensitive to ITIM–dependent signalling (50). Furthermore, KIR engagement by HLA-C on target cells inhibits LFA-1–dependent adhesion (51). Phosphorylation of co-activation receptors 2B4 and NKG2D, and their recruitment to lipid rafts, are blocked by KIR binding to HLA-C on target cells (52,53). Altogether, these results have led to a new model for activation and inhibition of

NK cell responses to target cells (Fig. 3). By blocking actin-dependent processes, such as receptor clustering and coalescence of lipid rafts into larger signalling platforms, inhibitory KIR achieves inhibition at a very early step. This model can account for the ability of KIR to prevent NK cell activation by a large variety of receptors and signalling pathways. Placing ITIM-bound SHP-1 upstream of the full-strength signalling by clustered activating receptors is a better strategy for inhibition than to have SHP-1 chase after phosphorylated receptors and signalling components.

The signalling complex, or "signalosome", to which Vav1 is associated at the time of its dephosphorylation by SHP-1 remains to be identified. As both Vav1 and its GTPase effector Rac1 are multifunctional proteins, many potential interactions with signalling molecules exist. As inhibition of NK cells by engagement of KIR blocks activation upstream of Ca^{2+} flux, one can expect that $Ca2^{+}$–dependent degranulation would be inhibited. It will be interesting to test whether ITIM–mediated inhibition can also prevent granule polarization, given that polarization and degranulation are controlled by separate signals (54).

The extended ITIM family

Receptors and cells

Since the original description of the ITIM sequence and, in part due to the value of the ITIM as predictor of inhibitory function, many ITIM-Rs have been discovered, some of which belong to their own subfamily within the larger ITIM-R superfamily. A stringent search for potential ITIM-Rs in the human proteome has identified 109 candidates, of which only 36 have been described previously as inhibitory receptors (55). ITIM-Rs exert their control in a very wide range of cell types and regulate diverse functions (Table 1). Specific recruitment of SHP-1 and SHP-2 to phosphorylated ITIM has been confirmed repeatedly. However, evidence that SHP plays a role in ITIM-R function is still missing in most cases. Binding of SHP to an ITIM does not imply that SHP is necessary for function, as shown with FcγRIIb (14). While selective binding of either SHP-1 or SHP-2 is often observed with ITIM-Rs, the functional significance of this selectivity is unclear.

In vivo analysis of ITIM-R function through genetic ablation of genes encoding ITIM-Rs has provided some striking examples of the importance of this negative regulatory system (Table 1). Ablation of the programmed cell death 1 (PD-1) gene results in spontaneous autoimmunity (56). PD-1 binding to its ligands (PD-L) on antigen presenting cells induces peripheral tolerance, whereas binding to PD-L on parenchymal cells prevents tissue destruction (56). $CD8^{+}$ T cell tolerance is controlled by the combination of CTLA-4 and PD-1 binding to their ligands on dendritic cells (57). Negative signalling by PD-1 can also be detrimental, as it causes exhaustion of $CD8^{+}$ T cells during chronic virus infection (58). This exhaustion can be reversed by blockade of the PD-1–PD-1L interactions. Yet another role of PD-1 is to promote feto-maternal tolerance by restraining alloreactive T cells through binding to PD-L1 expressed in decidua basalis (maternal tissue in contact with the fetal trophoblast) (59). The B and T lymphocyte attenuator (BTLA) is another ITIM-R, expressed in B and T cells, which collaborates with PD-1 to achieve tolerance (60,61). BTLA-deficient lymphocytes are hyper-responsive to antigen-receptor signals.

PIRB is an ITIM-R in mice, which is expressed in B and myeloid cells, and is considered an ortholog of human LILR. PIRB and LILR bind MHC class I molecules. $PIRB^{-/-}$ mice have augmented Th2 responses (62), and accelerated graft versus host disease (GvHD) due to higher DC activation upon transfer of allogeneic splenocytes (63). Strikingly, mutant mice lacking functional PIRB exhibit more robust cortical ocular-dominance plasticity, demonstrating an important function of an ITIM-R in the central nervous system (64). Expression of PIRB in several other regions of the brain suggests that it may function broadly to stabilize neural

circuits. A specific function of the ITIM-R SIRPα was revealed in SIRPα$^{-/-}$ mice, in which red blood cells were rapidly cleared from the blood. Red blood cells are normally protected from phagocytosis by macrophages through expression of CD47, a ligand of SIRPα (65).

Regulation by ITIM-Rs can be complex. For example, CD22, which is a member of the Siglec receptor family, is an ITIM-R associated with membrane immunoglobulin on B cells. Upon BCR ligation and signalling, CD22 becomes phosphorylated and recruits SHP-1, which dampens the BCR signals. However, independent ligation of CD22 releases the BCR signals from inhibition by CD22-associated SHP-1, thereby lowering the activation threshold for B cell activation (66). Therefore, signalling by an ITIM-R does not necessarily result in inhibition of cellular activation. Another complex mode of BCR regulation by CD22 has been revealed: Tyrosine phosphorylated CD22 attenuates Ca^{2+} responses by promoting plasma membrane calcium-ATPase (PMCA) activity (67). PMCA extrudes cytoplasmic Ca^{2+} through the plasma membrane, thereby blocking BCR activation signals. The SHP-1–dependent activation of PMCA, and the association of PMCA with CD22, suggest that PMCA may be activated through dephosphorylation by SHP-1 (67). Additional examples of the diverse ITIM-R functions are listed in Table 1.

ITIM-Rs often belong to small families of closely related members with similar extracellular domains but shorter cytoplasmic tails. Some of the receptors lack ITIM sequences in their tails and transmit positive signals through association with ITAM-containing subunits. The diversification of inhibitory receptor families to include allelic polymorphism and activating isoforms may have been driven by selection for resistance to pathogens. Indeed, activating members in both KIR and Ly49 receptor families have evolved more recently from ancestral inhibitory KIR and Ly49 receptors (68). Activating KIRs contribute to disease susceptibility and to resistance to pathogens, as shown by studies of genetic linkage between KIR and HLA haplotypes (69). Another example of the potential advantage of receptor diversity, including inhibitory and activating forms, is illustrated by genetic resistance to the mouse Herpes virus CMV (MCMV). Among its many strategies to evade immune responses, MCMV expresses the MHC class I-like molecule m157, which binds to the inhibitory receptor Ly49I (70). Expression of m157 compensates for the inhibition of host MHC class I expression by other MCMV proteins. However, C57BL/6 mice are resistant to MCMV because m157 is recognized by the activating isoform Ly49H (70,71).

Although the ITAM-associated members of ITIM-R families must have important functions, as suggested by their evolution under strong selective forces and by genetic studies of their disease associations, some common misconceptions about their role should be addressed here. The ITIM-containing and ITAM-associated members of these receptor families are sometimes referred to as “paired” receptors. However, it is clear that there is no obligate pairing, either in their expression or function. Healthy individuals with normal NK cell cytotoxic function but with a complete lack of activating KIRs are fairly common. Activating isoforms of ITIM-R families in NK cells (e.g. KIR2DS, Ly49H, CD94/NKG2C) are clearly dispensable in the activation of NK cell cytotoxicity. Furthermore, ITIM-containing receptors are not limited to blocking activation signals delivered by ITAM-associated receptors only.

An outstanding question about most of the ITIM-Rs is how they signal, and whether the ITIM-bound SHP-1/2 is always important for receptor function. Is there a common inhibitory mechanism used by all or most ITIM-Rs? For many of the ITIM-Rs described so far, there is only limited understanding of the negative signalling pathway involved.

Signalling by ITIM-Rs

The integration of signals transmitted during contact between cells through the engagement of several activating and inhibitory receptors results in a finely tuned cellular response. Therefore,

when evaluating receptor function, it is critical to use experimental approaches that preserve the physiological situation under which those receptors are engaged (72). Receptor crosslinking with antibodies eliminates the unique environment of cell contacts and the formation of immune synapses. The use of antibodies in solution, on solid supports, or in redirected assays with FcR^+ cells, creates artificial conditions in which receptors are bound with an affinity that is several orders of magnitude greater than the binding affinity of natural ligands.

There are cases where ITIM-R crosslinking with antibodies has led to erroneous conclusions about their function. For instance, antibody co-crosslinking of CD22 with BCR inhibits BCR-induced signals. However, CD22 engagement by its natural ligand releases the inhibitory effect of CD22 on BCR signals by separating CD22 from BCR clusters (66). Redirected assays using soluble antibodies to engage an ITIM-R, and FcR^+ cells to obtain a multivalent interaction, can be used to evaluate the inhibitory potential of a given ITIM-R, but are not suited to draw conclusions about ITIM-R function. Therefore, studies of signalling properties and receptors function should be carried out in the natural situation of receptor–ligand interactions during contact between cells.

In NK cells, SHP is required for inhibition by an ITIM–containing KIR. Vav1 is the likely substrate of SHP-1 during the inhibition exerted through KIR binding to HLA class I on target cells. It is not known whether this mechanism for inhibition applies to other ITIM-Rs. However, the general concept of a single, or a few molecules that are selectively targeted for dephosphorylation by SHP may apply to inhibition by other ITIM-Rs on NK cells or other cells. Substrate selection during inhibition is most likely dictated, not by sequence preference of the SHP catalytic site, but by substrate accessibility in space and time.

An experimental approach to identify SHP substrates is to monitor protection of tyrosine-phosphorylated proteins through expression of catalytically inactive mutants of SHP. Using this approach in cells that were stimulated by engagement of activation receptors, but without engagement of inhibitory receptors, CD72, actin, and myosin were identified as SHP substrates in B cells (73–75). As phosphorylated CD72 promotes growth arrest and apoptosis, dephosphorylation by SHP blocks this negative regulation. By the same approach, IgM crosslinking led to the association of SLP76 with CD22, and protection of SLP76 phosphotyrosines by a catalytically inactive SHP (76). CD22 may therefore recruit substrates for dephosphorylation by CD22–associated SHP.

Inhibition through an ITIM-R can also be independent of SHP-1 and SHP-2. Phosphorylated LAIR-1 binds the tyrosine kinase Csk, which is a negative regulator of Src-family kinases. Furthermore, co-crosslinking of LAIR-1 and BCR in DT40 cells inhibited BCR-induced activation, even in the absence of SHP-1 and SHP-2. In those cells, LAIR-1 bound Csk but not SHIP, implying a role of Csk in the inhibitory signal (77). The ITIM-Rs ILT2 and SIRPα also bind Csk (Table 1).

The phosphorylation status of different ITIM-Rs varies. Some ITIM-Rs, such as PIRB, are constitutively phosphorylated (78). The kinases responsible for PIRB phosphorylation in DC and neutrophils are the Src-family kinases Hck and Fgr (79). SHP-1 is constitutively associated with phosphorylated LAIR-1 in T cells (80). In addition, tyrosine residues outside of ITIM sequences can modulate ITIM phosphorylation, as shown in ILT2 (81). Although SHP-1 and SHP-2 are interchangeable for inhibition by the PIRB cytoplasmic tail in DT40 B cells (82), these two phosphatases have interesting differences in their properties. Considering that preferential association with either SHP-1 or SHP-2 occurs with a number of ITIM-Rs, differences in negative signalling between SHP-1 and SHP-2 could be relevant for ITIM-R function. SHP-2 contributes to several activation pathways, independently of its ability to bind phosphorylated ITIMs (83,84). Unique structural features suggest that recruitment of SHP-1

may have a different outcome on signalling than recruitment of SHP-2. Binding of SHP-1 to ITIM-Rs requires phosphorylation of two ITIM sequences. Upon recruitment, SHP-1 becomes phosphorylated at two C-terminal tyrosines. Each one of these tyrosines has the potential to form an intramolecular interaction with an SH2 domain (Fig. 4) (85). The spacing between the C-terminal tyrosines in SHP-1 is too short to allow simultaneous binding to the two SH2 domains (85). Therefore, SHP-1 should be most active when bound to the ITIM-R, as dissociation results in two, mutually exclusive, monovalent SH2 domain–phosphotyrosine interactions, one of which is catalytically inactive.

In contrast, the longer spacing between the C-terminal tyrosines of SHP-2 is compatible with intramolecular engagement of both tyrosines with the tandem SH2 domains (86). Therefore, SHP-2 has the potential to be released from the ITIM-R in a catalytically active conformation. Furthermore, as SHP-2 can bind to inhibitory KIR that is phosphorylated at the first ITIM only, release of SHP-2 in its divalent intra-molecular form may be favoured over the monovalent, intermolecular association with the ITIM-R (Fig. 4). Even a transient association of SHP-2 with a phosphorylated ITIM, together with phosphorylation of both C-terminal tyrosines in SHP-2, may result in release of SHP-2 with sustained catalytic activity. Therefore, SHP-2 has the potential to dephosphorylate molecules while it is no longer bound to the ITIM-R. The functional consequences of these different properties of SHP-1 and SHP-2 have yet to be tested.

Inhibition by “pseudo”-ITIM receptors

Among the many receptors with inhibitory function, some recruit SHP-1 through phosphorylated tyrosines that are not within typical ITIM sequences (Table 2). It is worth describing what is known about their inhibitory mechanism, as it may shed light on how SHP recruited by *bona-fide* ITIM-Rs may inhibit activation signals. Early on, it was shown that signalling by the erythropoietin receptor (EPO-R) was terminated by recruitment of SHP-1 to the phosphorylated tyrosine at position 429, and dephosphorylation of the EPO-R–associated kinase JAK2 (87). This type of inhibition, namely the termination of a signal delivered by the receptor to which SHP-1 is recruited, is quite different from the function of ITIM-Rs, which prevent other activation receptors from signalling at all. Inhibition of signals from the EPO-R and the prolactin receptor through SHP-1 can also occur through recruitment of a complex of the suppressor of cytokine signalling-1 (SOCS-1) and Grb2 by SHP-1 (88). In this case, inhibition is independent of the SHP-1 catalytic activity.

The death receptors Fas, TNF-R, and TRAIL bind SHP-1, SHP-2, and SHIP through a YxxL motif in their cytoplasmic tail (89). Signaling by these death receptors prevents GM-CSF–induced activation of the Src-kinase Lyn. Presumably, dephosphorylation of Lyn by SHP-1 blocks the anti-apoptotic signalling pathway induced by GM-CSF. Additional examples of inhibition by non-ITIM receptors are listed in Table 2.

Two receptors involved in inhibition of T cell activation, namely CTLA-4 and CD5, had been proposed to inhibit by a mechanism similar to that of ITIM-Rs. However, CTLA-4 inhibitory function is tyrosine-independent (90), and the inhibition of Ca^{2+} flux, Erk activation, and cytokine production by CD5 depends on a cytoplasmic tyrosine that does not bind SHP and SHIP (91).

Surprisingly, there are examples of signalling by ITAM-containing receptors that result in inhibition. The high affinity IgA receptor FcαRI, which is associated with the ITAM-containing FcR γ chain, inhibits signalling by FcγR or FcεRI when bound to soluble IgA (92). The interaction of FcαRI with low avidity monomeric IgA results in recruitment of SHP-1, and inactivation of the early signalling components, Syk, LAT, and Erk (92). This inhibitory property of ITAM could explain how TLR signalling is enhanced in the absence of ITAM-based signals in macrophages (93). Inhibition of TLR responses occurs through DAP12–

associated receptors, such as TREM2 (94,95). These findings provide a new framework for understanding how upregulation of DAP12–associated LIR7 in lesions of lepromatous patients had a suppressive effect towards TLR-mediated antimicrobial activity (96).

Regulation of NK cells by a complex repertoire of inhibitory receptors

The repertoire of inhibitory receptors on NK cells is complex at several levels. First, three families of MHC class I-specific inhibitory receptors are expressed in human (KIR, CD94/NKG2, LILR) and mouse (Ly49, CD94/NKG2, PIR) NK cells. Second, expression of receptors in the KIR, Ly49, and CD94 families is turned on in a stochastic manner, such that each NK cell expresses its own repertoire of receptors. Third, there is very high genetic diversity in the KIR and Ly49 receptor families. KIR genes vary in number, genomic organization, and allelic polymorphism among individual haplotypes. This combination generates diversity such that most individuals have a unique KIR repertoire (97). And finally, NK cells express other inhibitory receptors that bind non- MHC class I ligands such as NKR-P1, LAIR-1, Siglec-7/9, and CEACAM1 (Table 1). It is not known yet if this multiplicity of inhibitory receptor–ligand systems serves to protect different types of cells, which may express only a subset of inhibitory receptor ligands. Alternatively, the additive effects of several inhibitory receptor–ligand interactions may be required to achieve full inhibition. Another hypothesis is that different inhibitory receptors may specialize in the type of signals and cellular function they control.

MHC class I recognition by inhibitory receptors is crucial for protection of healthy cells from NK cells. For example, CD94/NKG2A binding to Qa1 on activated $CD4^+$ T cells is essential for their protection from NK cell-mediated lysis (98). However, NK cell tolerance develops in the complete absence of MHC class I (99,100). This tolerance towards MHC class I-negative cells is actively maintained, and is quickly lost when tolerant NK cells are mixed with MHC class I-positive cells (101,102). Blocking of the inhibitory Ly49–MHC class I interaction in vivo with antibodies interfered with the development of a functional NK cell population (103). NK cells that lack inhibitory receptors for self MHC class I exist but are hypo-responsive (104). The intrinsic responsiveness of NK cells is calibrated by an ITIM-dependent process (105). NK cells that receive stronger inhibitory signals through MHC class I recognition acquire a stronger capacity to respond to activation signals (105,106). An interesting implication is that genetic associations between combinations of KIR and HLA haplotypes with disease could be accounted for, not only on the basis of inhibition by ITIM-Rs, but also by the calibration of NK cell responsiveness acquired through engagement of ITIM-Rs. It should be noted that these two properties of ITIM-Rs have opposite effects. For example, the combination of an inhibitory KIR and its cognate HLA-C can, on the one hand, provide inhibition during contact with target cells, but, on the other hand, confer greater intrinsic ability to respond to activation signals. Perhaps such opposite effects will explain the complex patterns of disease associations with ITIM-Rs that have emerged in genetic studies (69).

Concluding remarks

The prominent role of inhibitory receptors in the maintenance of a balanced immune system has been illustrated vividly with mice that are deficient in selected receptors of the ITIM-R superfamily. Identification of a vast array of specific inhibitory receptor–ligand combinations, and expression of those ligands in diverse cell types, has revealed a sophisticated system for the regulation of cellular responses in various environments. An important concept to emerge from studies of inhibitory signalling during NK cell contact with resistant target cells, is that a single predominant component of the activation pathway is targeted for dephosphorylation by SHP-1. That inhibition is not mediated simply by dephosphorylation of many signalling components in the activation pathway, but by the selection of a specific substrate for SHP, suggests that ITIM-Rs may be capable of a finely tuned regulation of activation signals.

However, the orchestration of inhibitory signals and their intersection with specific activation signals are still unknown for most of the ITIM-Rs. A key challenge that lies ahead is to determine in molecular detail the initiation and propagation of inhibitory signals delivered by different ITIM-Rs in their natural physiological contexts.

Acknowledgements

I thank Sumati Rajagopalan for comments on the manuscript. Supported by the Intramural Research Program of the NIH, NIAID.

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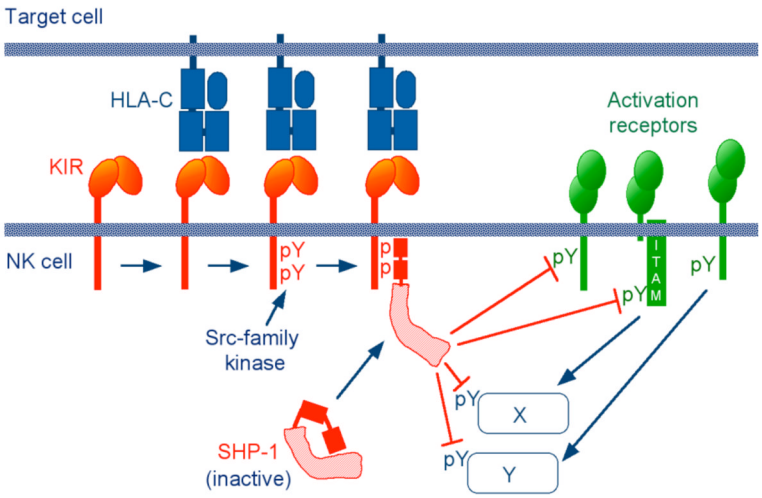

Fig. 1. Early model for inhibitory signalling by KIR in NK cells

Sequential steps in the inhibition of NK cells by KIR: Binding of inhibitory KIR to HLA-C on target cells; KIR clustering; phosphorylation of two tyrosines within cytoplasmic ITIM sequences; recruitment and activation of the tyrosine phosphatase SHP-1 to the tyrosine-phosphorylated ITIMs; dephosphorylation of multiple substrates, such as activation receptors and signalling molecules (*X*, *Y*) by catalytically active SHP-1. The Src-family kinase that phosphorylates the ITIMs may be provided in *trans* by activation receptors. Inhibitory molecules are indicated in *red*, activation receptors in *green*.

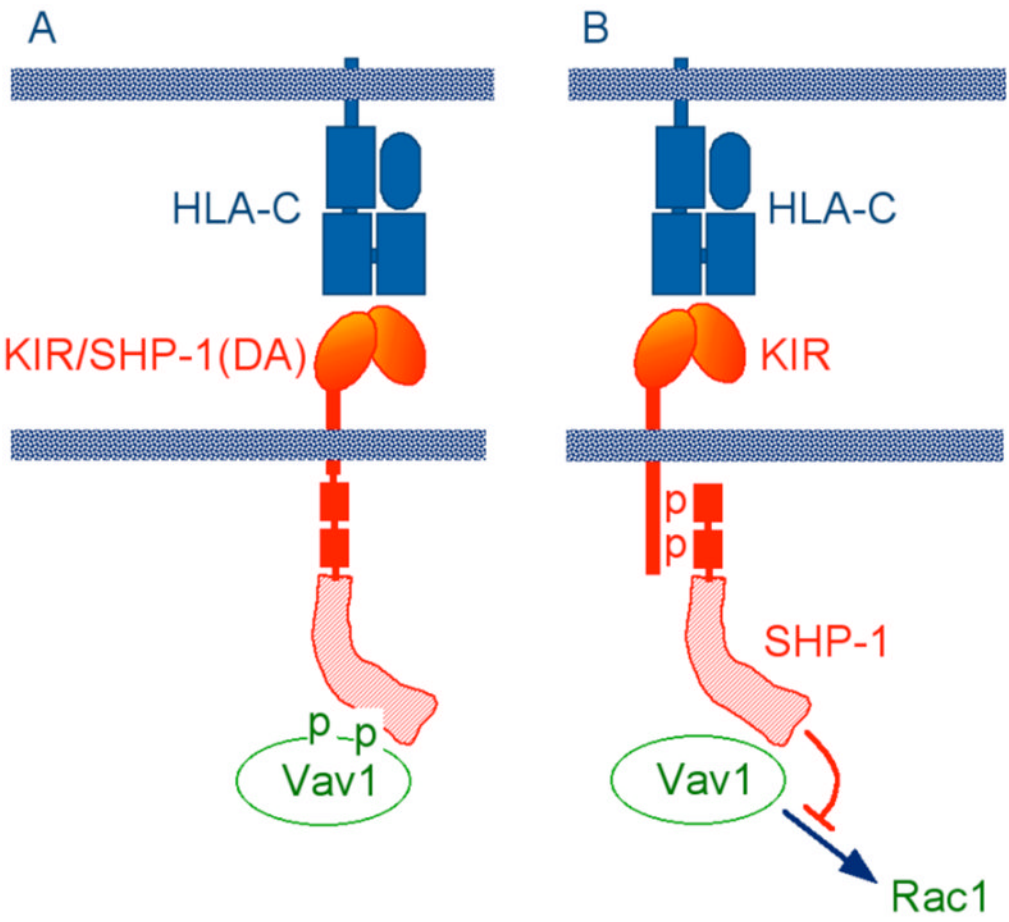

Fig. 2. Identification of Vav1 as the predominant substrate during inhibition of NK cells by KIR

(A) Tyrosine-phosphorylated Vav1 was "trapped" by a chimeric KIR/SHP-1 receptor during inhibition of YTS NK cells by target cells expressing an HLA-C ligand of KIR. The trap was generated by an Asp to Ala mutation (DA) in the SHP-1 catalytic site, and by the fusion of SHP-1(DA) to the KIR cytoplasmic tail. (B) Vav1 trapping, as shown in panel *A*, implies that catalytically active SHP-1 recruited by KIR during inhibition blocks NK cell activation through dephosphorylation of Vav1, which prevents the guanine exchange factor activity of Vav1 towards the GTPase Rac1.

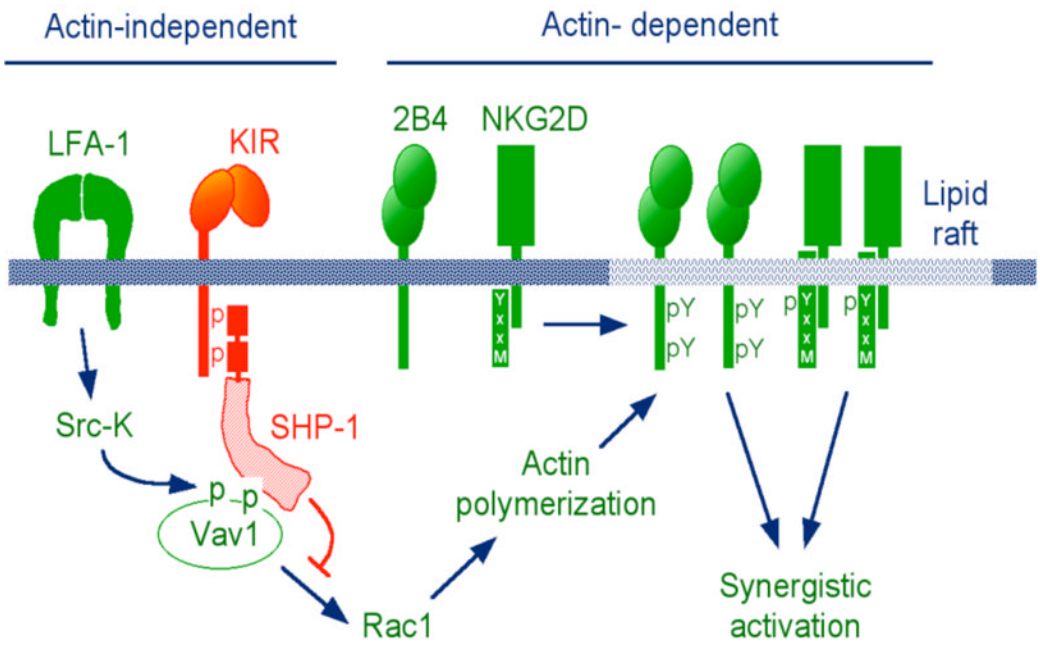

Fig. 3. Revised model for inhibitory signalling by KIR in NK cells

Early, actin-independent signalling by LFA-1 phosphorylates and activates Vav1. Actin-independent dephosphorylation of Vav1 by ITIM–bound SHP-1 prevents actin-dependent processes, such as recruitment of natural cytotoxicity receptors (e.g. NKG2D and 2B4) to lipid rafts, receptor tyrosine phosphorylation, and synergistic signalling by co-activation receptors.

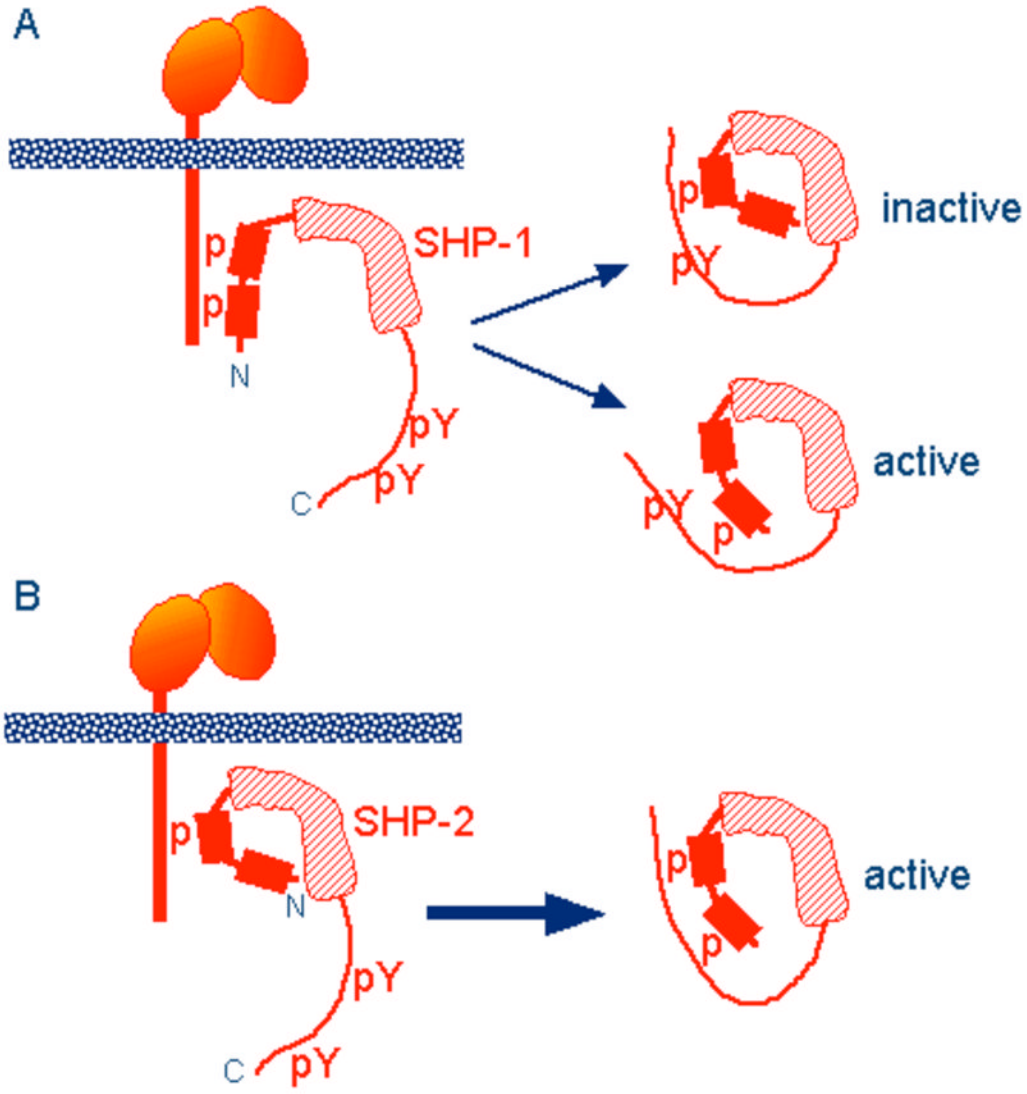

Fig. 4. Distinct structural properties of SHP-1 and SHP-2 suggest different inhibitory potential

These diagrams are adapted from (86) and (85). (A) The preferential binding of phosphorylated ITIM to the second SH2 domain of SHP-1 (1,4), and the crystal structure of SHP-1 (107) suggest that the first ITIM of inhibitory KIR binds to the second SH2 domain of SHP-1. The two C-terminal tyrosines of SHP-1 can engage in intramolecular interactions with the SH2 domains, when phosphorylated. However, the short spacing (28 amino acids) between the tyrosines preclude intramolecular binding to both SH2 domains simultaneously. (B) SHP-2 phosphorylated at both C-terminal tyrosines (38 amino acids apart) can form a divalent, intramolecular complex with its own SH2 domains, which retains catalytic activity. Unlike SHP-1, which requires phosphorylation of both ITIMs for binding, SHP-2 binds to either both phosphorylated ITIMs or to the first phosphorylated ITIM only, as indicated.

Table 1

Expression, ligands, signaling properties, and function of selected ITEM-containing receptors (ITIM-R)

Receptor	Expression	Ligands		SHP substrate	Selected Examples	Refs
		Extracellular	Intracellular		Inhibition (activation)* of:	
FcγRIIb	B, mast, myeloid	Multivalent IgG	SHIP, SHP†		Sustained Ca^{2+} flux, FcR signaling	(108,109)
KIR‡ (human) (CD158)	NK,T§	MHC class I (MHC-I)	SHP	Vavl	Early Ca^{2+} flux, Tyr-phosphorylation, cytotoxicity, cytokine secretion β_2 integrin-mediated adhesion	(28,47,110) (111) (112) (51)
Ly49‡ (mouse)	NK,T	MHC-I	SHP		NK cell activation	(7,18)
CD94/NKG2A‡	NK,T	HLA-E (human), Qal (mouse)	SHP		NK cell activation Lysis of $CD4^+$ activated T cells	(113,114) (98)
NKR-P1 (CD161)	NK,T	LLT1 (human) Ocil/Clrb (mouse)	SHP		NK cell activation	(115,116) (44,117,118)
LILR‡ (human) (ILT, LIR, CD85)	Broad	MHC-I	SHP, Csk		NK and T cell activation, T cell stimulation by DC	(42,119) (120)
PIRB‡ (mouse)	Broad, including brain	MHC-I	SHP		BCR and FcεRI signaling, DC maturation, integrin signaling, GvHD, neuronal plasticity	(62,121) (122) (63,64)
Kirl2 (mouse)	Regions of neuro-genesis in brain					(123)
SIRPα‡ (CD172a)	Myeloid	CD47, SP-A/D	SHP, Csk, Pyk2, Grb2, etc.		Cell growth, phagocytosis, adhesion	(65,124–126)
PD-1	T	PD-1L	SHP		Autoimmunity, anti-viral CDS T cell responses	(56) (58)
BTLA	B, T		SHP		Autoimmunity, proliferation	(60,61)
CD22 (Siglec-2)	B	Sialic acids	SHP, Grb2, SLP76	SLP-76	BCR basal signals, (Ca^{2+} efflux)	(66,76,127) (67)
Siglecs‡ (CD33 and CD33-related)	Broad	Sialic acids	SHP, SOCS3		Proliferation, adhesion	(128,129)
CD72	B	CDS	SHP, Grb2	CD72	Complex regulation of BCR signaling	(73)
LAIR-1	Broad	Collagen	SHP, Csk			(77,130)
KLRG1	NK, T, mast	Cadherins	SHP, SHIP		NK cell anti-viral response, TCR signaling	(131-134)
gp49B (mouse)	Mast, NK, T neutrophils, eosinophils	$\alpha v\beta_3$ integrin	SHP		Allergy, T cell activation, intravascular neutrophil adhesion, eosinophilia	(135,136) (137) (138)
PECAM-1 (CD31)	Platelets, T, endothelium, others	PECAM-1	SHP		TCR signaling, (NK cell transmigration, endothelial cell motility, integrin-mediated adhesion)	(139-142) (143) (144) (145)
CEACAM-1 (CD66a)	Broad	CEACAM-1/5/6/8, CD62E, galectin-3	SHP		NK and T cell activation, tumor cell growth	(146,147) (148)
TLT-1	Myeloid, platelets		SHP		(Ca^{2+} flux)	(149)
CD300a/f (IRp60, IREM-1/2)	Myeloid, NK		SHP, PI3K		NK cell activation, eosinophil activation	(150-152)
CLM-1 (mouse) (CMRF-35-like)	Myeloid		SHP		Osteoclastogenesis	(153)
PILRα‡	Myeloid	CD99	SHP			(154,155)

Receptor	Expression	Ligands		SHP substrate	Selected Examples	Refs
		Extracellular	Intracellular		Inhibition (activation)* of:	

*Activating signals of ITIM-Rs are given in parentheses.

†Includes SHP-1 and SHP-2. Preferential association with SHP-1 or SHP-2 occurs with some ITIMs.

‡Receptor family, which includes activating and inhibitory receptors.

§Some T cells, primarily those in the memory subset and IL-15-activated intra-epithelial lymphocytes, express NK cell receptors.

Table 2

Inhibition by non-ITEM and pseudo-ITEM containing receptors

Receptor	Expression	Ligands		SHP substrate	Inhibition (activation)* of	Refs
		Extracellular	Intracellular			
CTLA-4	T	CD80, CD86			T cell activation	(156)
CDS	B,T	CD72, other			Ca^{2+} flux, Erk2, cytokine production	(91,157)
GF-R†	Broad	Growth factors	SHP‡	JAK	JAK-Stat signaling	(87,88,158)
IL-4Rα	Broad	IL-4	SHP, SHIP		Stat6, IL-4 induced proliferation	(159)
TNF-R family	Broad	TNF-R ligands	SHP, SHIP	Lyn	(Apoptosis), anti-apoptotic signals	(89)
CD47	Hemopoietic	SIRPα, thrombospondin			IL-12R, Thl response (Actin, PKCθ)	(160) (161)
CD200R	Myeloid, mast	CD200	Dokl/2		MAPK, autoimmunity, degranulation	(162–164)
FcαRI	Myeloid	Soluble IgA	SHP		Syk, LAT, Erk, phagocytosis, allergy	(92)
DAP 1 2-associated receptors	Broad	Multiple			TLR signals, macrophage activation, antimicrobial defense	(94–96)

*Activating functions are given in parentheses.

†Growth factor receptors (e.g. EPO-R, PDGF-R).

‡Includes SHP-1 and SHP-2. Preferential association with SHP-1 or SHP-2 occurs with some ITIMs.