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How ITAMs Inhibit Signaling

Lionel B. Ivashkiv*

Arthritis and Tissue Degeneration Program, Hospital for Special Surgery, Immunology and Microbial Pathogenesis Program, Weill Cornell Graduate School of Medical Sciences, 535 East 70th Street, New York, NY 10021, USA

Abstract

Immunoreceptor tyrosine-based activation motifs (ITAMs) are used by multiple receptors to activate immune cells. However, ITAM-associated receptors can have paradoxically inhibitory effects, which have been implicated in regulation of inflammatory responses, but mechanisms of inhibitory signaling are poorly understood. New evidence shows that low avidity ligation of the ITAM-associated immunoglobulin A receptor Fc α RI (transient engagement of small numbers of Fc α RI) results in translocation of Fc α RI and the associated inhibitory Src homology 2 (SH2) domain-containing phosphatase-1 (SHP-1) to membrane lipid rafts. Subsequent ligation of activating receptors results in their colocalization with Fc α RI and SHP-1 and trafficking to an inhibitory intracellular compartment termed the inhibisome. Thus, ITAM suppressive signals subvert the activating function of rafts to promote incorporation of receptors into supramolecular domains where signaling molecules are deactivated by SHP-1.

Immune cell activation and differentiation are tightly regulated by the balance between opposing signals generated by activating and inhibitory receptors. Inhibitory receptors serve to maintain cell quiescence under physiological conditions, fine-tune the amplitude of cell responses to be appropriate for the microenvironment, and prevent toxicity associated with excessive cell activation and secretion of inflammatory mediators (1). An emerging paradigm is that classically activating immunoreceptor tyrosine-based activation motif (ITAM)-associated receptors also paradoxically transmit inhibitory signals. The study by Pfirsch-Maisonnas *et al.* (2) substantially extends our understanding of inhibitory mechanisms used by ITAMs and explains how ITAMs broadly inhibit multiple heterologous receptors. Differences in mechanism and specificity of inhibition compared with those of conventional inhibitory receptors suggest distinct biological roles for different classes of inhibitory receptors.

A well-established paradigm in immune cells is that inhibition of signaling by activating receptors is mediated predominantly by inhibitory receptors harboring an immunoreceptor tyrosine-based inhibition motif (ITIM) in their cytoplasmic domain (1). Engagement of ITIM-containing receptors results in Src family kinase-mediated phosphorylation of ITIM tyrosine residues and recruitment of inhibitory signaling molecules, most prominently the Src homology 2 (SH2) domain-containing inositol poly-phosphate 5-phosphatase (SHIP) and SH2 domain-containing phosphatases-1 and -2 (SHP-1 and SHP-2). These phosphatases can then dephosphorylate active signaling molecules and thereby down-regulate signaling cascades.

ITIM-containing receptors recognize various ligands that are typically present on cell surfaces or in large extracellular aggregates. For example, natural killer (NK) cell KIRs

*ivashkivl@hss.edu.

(killer cell immunoglobulin-like receptors) recognize major histocompatibility complex (MHC) class I molecules, Siglecs recognize sialylated cell surface proteins, and FcγRIIb recognizes the Fc region of immunoglobulin G (IgG) antibodies and binds immune complexes (1). Several ITIM-containing receptor ligands are broadly distributed on mammalian (host) cells and function to prevent inappropriate cell activation. Thus, KIRs prevent activation of NK cells by healthy host cells that have cell-surface MHC molecules, whereas decreased abundance of MHC class I in virally infected or tumor cells results in unopposed signaling by activating NK receptors, NK cell activation, and killing of target cells (1). One principle that has emerged is that ITIM-containing receptors typically recognize distinct ligands that are codistributed on cell surfaces or large extracellular complexes with ligands that are recognized by activating receptors. This joint recognition of targets present on the same surface results in coaggregation of ITIM-containing and activating receptors, which brings ITIM-associated phosphatases into close proximity to their targets and facilitates inhibition. Coaggregation also augments ITIM function through increased phosphorylation of ITIM tyrosines by kinases associated with activating receptors, thus establishing a functional coupling between activating and inhibitory receptors. Co-recognition of ligands provides a basis for specificity of inhibition: Only activating receptors that are coligated and aggregated with ITIM-containing receptors, based on specific dual receptor-ligand interactions, are targeted for inhibition (1). In the case of the inhibitory ITIM-containing FcγRIIb, coaggregation with B cell receptors (BCRs) by simultaneous binding of FcγRIIb and BCRs to different ligands present in immune complexes results in cross-inhibition of BCR signaling and attenuation of B cell activation (1). Coaggregation of FcγRIIb with activating Fc receptors such as FcγRIII by joint recognition of immune complexes blocks Fc receptor (FcR) signaling and cell activation (1) (Fig. 1A).

Another established paradigm is that immune receptors associated with an ITAM are potent activating receptors (3). High-avidity cross-linking of ITAM-associated receptors results in phosphorylation of ITAM tyrosine residues, recruitment and activation of the kinase Syk, and generation of signals leading to activation of calcium and mitogen-activated protein kinase (MAPK) pathways, nuclear factor (NF) κB, and inflammatory cell activation [reviewed in (4)]. Concomitant with initiation of signaling after multivalent cross-linking, ITAM-associated receptors traffic into lipid rafts, specialized membrane microdomains that are enriched in active signaling components and serve as a platform to generate activation signals (5). These rafts coalesce during signaling, and activated receptors subsequently traffic to endocytic compartments, where signaling can be amplified, and then either recycle to the plasma membrane or are targeted for degradation. It has become clear that monovalent, low-avidity ligation of ITAM-associated receptors generates an inhibitory signal (6–11). Although several mechanisms by which ITAM-associated receptors can inhibit signaling have been proposed (4), an important mechanism depends on ITAM signaling and is mediated by recruitment of SHP-1 or SHIP instead of Syk, possibly related to “incomplete” phosphorylation of ITAM tyrosine residues (7, 8, 10–12). SHP-1 can then cross-inhibit signaling by heterologous receptors by a dephosphorylation-mediated mechanism analogous to that used by ITIM-containing adaptors. This direct ITAM–SHP-1 inhibitory mechanism is potentially activated after monovalent ligation of FcαRI by its physiological ligand IgA or by anti-FcαRI Fab fragments and mediates important suppressive effects under inflammatory conditions in vivo (7, 8).

Monovalent and low-avidity ligation of ITAM-associated receptors, including FcαRI, cross-inhibits multiple heterologous receptors, including ITAM-associated receptors, Toll-like receptors (TLRs), tumor necrosis factor (TNF) receptor family members, and chemokine receptors (7). Because these receptors do not share regions of homology or structural similarity and use large numbers of distinct signaling adaptors and signaling molecules, a major question in the field has been to identify mechanisms by which ITAM-induced

inhibitory signals target heterologous receptors for inhibition. The study by Pfirsch-Maisonnas *et al.* (2) takes a large step toward answering this question and provides molecular insights into mechanisms by which ITAM-associated receptors inhibit signal transduction. The authors used a well-established and functionally validated system, Fc α RI-transfected RBL mast cells that express Fc ϵ RI, the high-affinity receptor for the Fc region of IgE antibodies. In this system, activating Fc ϵ RI signaling is triggered by prebinding cell surface Fc ϵ Rs with antigen-specific IgE (“sensitization”) followed by cross-linking using multivalent antigen. Inhibitory effects of Fc α RI are tested by adding its ligand IgA or by monovalent ligation of Fc α RI with a specific Fab A77 before Fc ϵ RI cross-linking. Previous work established that Fc ϵ RI signaling and functional cell responses are attenuated by Fc α RI preligation by a SHP-1–dependent mechanism (7, 8). Moreover, key results obtained in this cell line system were confirmed in this study and in previous work using primary cells relevant to physiological and pathophysiological inflammatory activation, such as human monocytes.

The authors found that monovalent ligation of Fc α RI induced translocation of Fc α RI, along with associated SHP-1, into lipid rafts, specialized membrane microdomains where activating receptors translocate after multivalent ligation. Assembly into lipid rafts was itself dependent on SHP-1 and its role in regulating actin dynamics. Thus, monovalent ligation of Fc α RI resulted in the enrichment of a signaling inhibitor, SHP-1, in a microdomain where various activating receptors are recruited and where SHP-1 “lies in wait” for activated receptors and signaling molecules to dephosphorylate. Indeed, on subsequent multivalent ligation, Fc ϵ RI translocated to the same lipid rafts, where it colocalized with Fc α RI and SHP-1 (Fig. 1B). Next, Fc α RI and Fc ϵ RI localized to large intracellular clusters located in a polarized location within the cell; this trafficking pattern differed markedly from the entry of Fc ϵ RI into endocytic vesicles in the absence of Fc α RI preligation. These polarized structures, termed inhibisomes, were enriched in Fc α RI, Fc ϵ RI, Lyn, and SHP-1 and were inferred to represent the site where signaling inhibition occurs.

These findings reveal that inhibitory ITAM signaling subverts lipid rafts from an activating function to instead become an inhibitory site that suppresses rather than promotes signaling by cross-linked or activated receptors. Activated receptors enter lipid rafts as part of the normal course of activation of signal transduction but become diverted from activating pathways into inhibisomes, where signaling is instead terminated, likely by dephosphorylation by SHP-1. These results identify a new intracellular compartment that specializes in inhibition of signal transduction and provide further support for the importance of cytoskeletal reorganization and supramolecular organization of signaling domains, similar to processes that occur during immune synapse formation (13). Multiple receptors use lipid rafts for signaling, and thus recruitment to rafts followed by trafficking to inhibisomes explains how ITAM-mediated inhibition can target various heterologous receptors for inhibition.

There are several interesting differences between ITIM- and ITAM-mediated cross-inhibition of signaling by heterologous receptors (Fig. 1). Mechanistically, ITIM-mediated inhibition tends to exclude targeted receptors from lipid rafts, thereby limiting their access to activators and amplifiers of signal transduction. Instead, ITAM-mediated inhibition alters the function of rafts to deactivate entering receptors and shunt them to an intracellular compartment specialized for inhibition of signaling. ITIM-containing receptors selectively target heterologous receptors on the basis of coaggregation of receptors by joint recognition of specific ligands present on cell surfaces or large or insoluble extracellular complexes. In contrast, ITAM-mediated inhibitory signaling targets receptors activated by soluble ligands and broadly inhibits multiple receptors that enter rafts. This suggests a different core function for these two inhibitory mechanisms. ITIM-associated receptors survey multivalent

surface-bound ligands in the environment and selectively regulate immune effector functions, while preserving the majority of cell responses to multiple environmental stimuli. ITAM-associated receptors respond to low-valency ligation by soluble extracellular ligands to induce a broad state of decreased responsiveness whose purpose may be to prevent toxicity from overactivation of cells and excessive production of inflammatory mediators.

This study raises several important questions for future research. One puzzling observation is that a 40- to 120-min preligation of Fc α RI, longer than typically needed for receptors to enter lipid rafts, is required for inhibition to occur. This suggests a potential requirement for de novo gene expression and protein synthesis, as has been observed in other systems where ITAM-associated receptors inhibit signaling (14). Such gene induction may be mediated by extracellular signal-regulated kinase, which is required for effective inhibition (6, 8) but was not detected in inhibisomes. Syk was observed on the periphery of inhibisomes, and it will be important to test whether Syk can play an inhibitory role because Syk mediates cross-inhibition of TLRs in similar systems (15). Increased understanding of the physiological role of broad ITAM-mediated inhibition would be provided by more complete characterization of the specificity of inhibition and identification of receptors that escape inhibition. For example, signaling by cytokines that use the Janus kinase-signal transducer and activator of transcription (Jak-STAT) pathway is not inhibited, and can actually be enhanced, by low-avidity ITAM ligation (16). Characterization of cell responses to Jak-STAT-activating cytokines after monovalent Fc α RI ligation could yield additional insights into the biological role of Fc α RI-ITAM-mediated inhibition. Lastly, it will be important to more fully characterize the molecular composition of the inhibisome, to determine the inhibisome's quantitative contribution to inhibition of signaling, and to determine the fate of signaling proteins after entry and dephosphorylation in inhibisomes.

Acknowledgments

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Fig. 1.

Contrasting mechanisms of inhibition of signaling by ITIM- and ITAM-containing receptors. **(A)** Model of classical ITIM-mediated inhibition. The inhibitory ITIM-containing FcγRIIb and the activating FcγRIII are coaggregated by joint recognition of IgG Fc regions in immune complexes. The tyrosine-phosphorylated ITIM in the FcγRIIb cytoplasmic domain recruits SHP (and in some ITIM-containing receptors SHP-1 and SHP-2), which inhibit signaling by dephosphorylating activating receptors and downstream signaling intermediates. PIP₂, phosphatidylinositol 4,5-bisphosphate; PI3K, phosphatidylinositol 3-kinase; P, phosphate; Y, tyrosine. **(B)** Model of ITAM-mediated inhibition. Dimerization of FcαRI by monomeric IgA or monovalent ligation by Fab fragments (not depicted) induces association of SHP-1 and translocation into rafts. Various receptors that typically use rafts for positive signaling localize to rafts after ligation by their respective ligands and thus colocalize with FcαRI and SHP-1. These include FcεRI, FcγRIII, TNF receptor, chemokine (CC-motif) receptor 2 (CCR2), and TLRs. Deactivation of receptor signaling by SHP-1 is likely initiated in rafts and is completed after translocation to an intracellular inhibisome compartment.