IMMUNOLOGY

HemITAM: A single tyrosine motif that packs a punch

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Innate immune cells sense danger through a plethora of germline-encoded receptors that recognize pathogen-associated molecular patterns (PAMPs) or cellular molecules that are exposed only by stressed, infected, malignant, or dead cells. Many of these danger-sensing receptors belong to the C-type lectin-like superfamily (CLSF) and therefore are called C-type lectin-like receptors (CTLRs). Certain activating CTLRs, namely, CLEC-2, Dectin-1, DNGR-1, NKp80, and NKp65, which are encoded by genes that are clustered together in a subregion of the mammalian natural killer gene complex (NKC), use a single copy tyrosine signaling module termed the hemi-immunoreceptor tyrosine-based activation motif (hemITAM). These hemITAM-bearing CTLRs are present on myeloid cells and innate lymphocytes and stimulate various functions, such as phagocytosis, cytokine production, and cytotoxicity. Proximal signaling mechanisms involve the tyrosine phosphorylation of the hemITAM and the subsequent activation of the kinase Syk. Signaling and Syk recruitment by the hemITAM appear to be tuned by variable amino acids within or near the hemITAM, which give rise to differences in downstream signaling events and diverging functional outcomes among hemITAM-bearing receptors.

Introduction

The mammalian immune system comprises multiple cell types with an adaptive branch represented by T and B lymphocytes and an innate branch including cells of both the myeloid and lymphoid lineages. Whereas the adaptive immune response is hallmarked by the generation of highly diversified antigen receptors through random somatic gene rearrangements and clonal selection, the innate immune response relies on germline-encoded receptors recognizing conserved molecular patterns of invading pathogens or molecules exposed by dead, infected, malignant, or stressed cells. T cell receptors (TCRs) recognize foreign peptides loaded onto major histocompatibility complex (MHC) class I and class II molecules and induce cytotoxicity or cytokine production, or both, by the two main classes of T cells, cytotoxic $CD8^+$ T cells and helper $CD4^+$ T cells, respectively. In contrast, B cell receptors (BCRs) bind to free antigens and trigger antibody production by the respective B cells. Initial activation of naïve T and B cells in response to ligation of the TCR and BCR critically depends on costimulatory signals provided by additional receptors such as CD28 or CD19, respectively.

Myeloid cells comprise macrophages, granulocytes, and dendritic cells (DCs) that sense pathogens or damage through a broad range of highly diversified receptors. Effector functions of myeloid cells involve the phagocytosis and subsequent destruction of invading pathogens as well as the production of cytokines and chemokines that induce and shape nascent immune responses. By processing and presenting foreign peptidic antigens with MHC molecules, DCs activate the adaptive immune system and thereby facilitate not only the elimination of pathogens but also the generation of a long-lasting and protective immune memory. Innate lymphocytes are subdivided into natural killer (NK) cell and tissue-resident innate lymphoid cell (ILC) populations. The latter are currently categorized into three groups according to their distinct cytokine profiles, which shape subsequent immune responses according to the site of infection and type of infectious agent (*1*). NK cells also shape immune responses through the secretion of cytokines such as interferon-γ (IFN-γ) and tumor necrosis factor (TNF) but, in contrast to ILCs, are also capable of exerting potent cytotoxicity toward stressed, infected, or malignant target cells. Cytotoxicity by NK cells and effector responses of myeloid cells can be enhanced by antibodies, because constant regions (Fc) of antibodies bound by Fc receptors allow for antibody-dependent cellular cytotoxicity or antibody-mediated phagocytosis, respectively. The multiple receptors endowing innate immune cells with the capability to sense and fight dangers can be categorized into several groups according to their structural framework. These classes include Toll-like receptors (TLRs) and NOD-like receptors (NLRs), as well as members of the C-type lectin superfamily (CLSF) and of the immunoglobulin-like superfamily (IgSF) that, upon sensing danger, relay signals into the respective innate immune cell through various mechanisms (*2*–*5*).

Tyrosine-Based Signaling Motifs in Immunoreceptors

Signaling by many transmembranous immunoreceptors of the IgSF and CLSF originates at cytoplasmic tyrosine-based motifs that are present either in the receptors themselves or within adaptor proteins that associate with the receptors. The most prominent and earliest-described motifs mediating intracellular signaling by these receptors are the immunoreceptor tyrosine-based activation motifs (ITAMs) and the immunoreceptor tyrosine-based inhibition motifs (ITIMs) (*6*–*9*). However, later studies defined additional motifs such as the hemi-immunoreceptor tyrosine-based activation motif (hemITAM), the immunoreceptor tyrosine-switch motif (ITSM), the immunoglobulin tail tyrosine (ITT) motif, and an ITT-like motif (*6*, *10*–*13*). The common mode of action associated with these motifs is the phosphorylation of the central tyrosine by Src family kinases (SFKs) creating a binding site for Src homology 2 (SH2)–containing proteins, which become activated upon recruitment. The core module of all these motifs consists of the amino acid sequence YxxΦ (Φ denoting amino acids L, V, I, M, or Y), with various types of motifs further defined by other particular amino acids within or adjacent to this core module (Fig. 1).

In contrast to all other tyrosine-based motifs, an ITAM consists of a tandem of two tyrosine core modules separated by a spacer of six to eight amino acids. ITAMs are not present in the cytoplasmic domains of the immunoreceptors themselves but are integrated into a few specialized transmembrane ITAM-bearing adaptor proteins such as CD3ζ, FcRγ, or DAP12, which tightly associate with the activating immunoreceptors through charged amino acids in their juxtaposed

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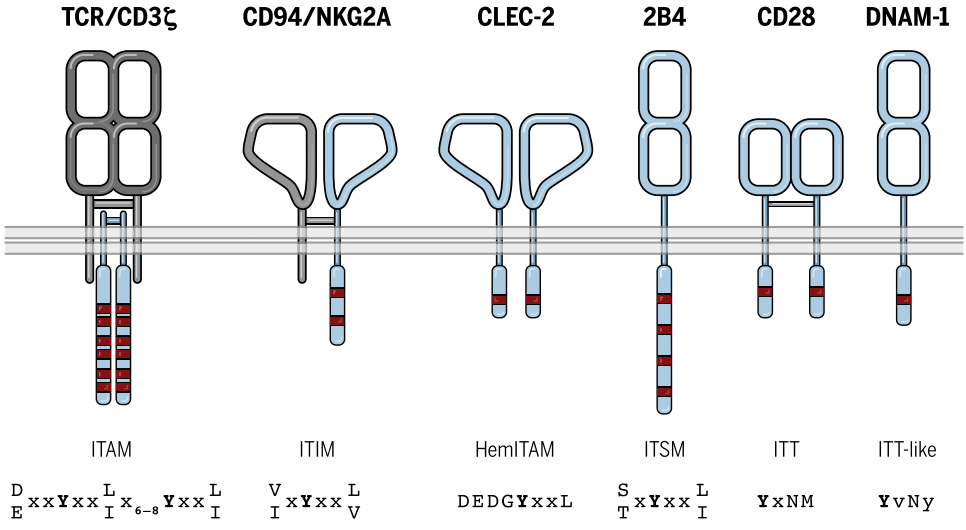


Fig. 1. Immunoreceptor-associated tyrosine-based signaling motifs. The tyrosine-based signaling motifs found in immunoreceptors include the immunoreceptor tyrosine-based activation motif (ITAM), the immunoreceptor tyrosine-based inhibition motif (ITIM), the hemi-immunoreceptor tyrosine-based activation motif (hemITAM), the immunoreceptor tyrosine-switch motif (ITSM), the immunoglobulin tail tyrosine (ITT) motif, and an ITT-like motif. The consensus sequence for each motif is shown below a cartoon depicting a prototypic receptor of each type, with "x" denoting any amino acid. The protein chains containing the signaling motif/motifs are indicated in blue, and the tyrosine core motifs are highlighted in red. These core tyrosine motifs are present as tandem copies in ITAMs and as single copies in the other motifs. Immunoreceptors that couple to ITAM- or ITIM-bearing polypeptides are indicated in gray. CD94/NKG2A and CLEC-2 are C-type lectin-like receptors (CTLRs); all others are members of the immunoglobulin-like superfamily.

transmembrane regions. Activating immunoreceptors associating with ITAM-bearing adaptors are mostly members of the IgSF such as the clonotypic antigen receptors (TCR and BCR), Fc receptors, and natural cytotoxicity receptors (NCRs), but there are also several type II transmembrane C-type lectin-like receptors (CTLRs), such as Nkrp1c and the heterodimeric receptor CD94/NKG2C, that associate with ITAM-bearing adaptors (*14*).

In contrast to ITAMs, ITIMs are located within the cytoplasmic domains of inhibitory immunoreceptors, consist of only one tyrosine module, and recruit phosphatases such as SH2 domain–containing phosphatase-1 (SHP-1) and SHP-2 or SH2 domain–containing inositol 5′ phosphatase–1 (SHIP-1) that efficiently counteract the kinase activity that is initiated by activating receptors (*3*). ITSM motifs were identified in the cytoplasmic regions of the signaling lymphocyte activation molecule (SLAM) family receptors, which are immunoglobulin-like type I transmembrane receptors that are present on cells throughout the hematopoietic system and are mostly engaged in homotypic interactions (*12*, *15*). Receptors of the SLAM family are characterized by their ability to recruit, through an ITSM, the SLAM-associated protein (SAP), which in turn recruits the SFK Fyn, leading to cellular activation (*16*–*18*). The absence of SAP, however, allows for recruitment of protein tyrosine phosphatases such as SHP-1 by SLAM family receptors, resulting in inhibitory signaling (*16*–*18*). ITT motifs are present in the cytoplasmic tails of certain membrane-associated immunoglobulins (for example, IgGs) and immunoglobulin-like costimulatory molecules such as CD28, as well as in the signaling adaptor DAP10 (*13*, *19*–*21*). Whereas ITT motifs recruit both the growth factor receptor–bound protein 2 (Grb2) and the p85 subunit of the phosphatidylinositol 3-kinase (PI3K) (*13*, *20*), ITT-like motifs were described as motifs recruiting either only Grb2, such as the ITT-like motif of DNAM-1, or only PI3K (*13*, *21*). ITSM, ITT, and the ITT-like motif all function as single copies of the tyrosine core module.

The term hemITAM was originally coined by Reis e Sousa and colleagues (*22*), referring to activating sequence motifs in the cytoplasmic domains of the myeloid CTLRs Dectin-1 and CLEC-2, which contain only a single tyrosine module and were shown to recruit Syk kinase upon tyrosine phosphorylation (*23*, *24*). Later studies reported similar activating hemITAMs in the cytoplasmic domains of the CTLRs DNGR-1 (*25*), NKp80 (*26*, *27*), and NKp65 (*28*, *29*), which are encoded together with Dectin-1 and CLEC-2 in close proximity to each other within the human NKC (Fig. 2A). Whereas the genes encoding CLEC-2, Dectin-1, and DNGR-1 have been formally assigned to the Dectin-1 (*CLEC7A*) gene cluster (*10*, *22*, *30*, *31*), NKp80 (*KLRF1*) and NKp65 (*KLRF2*) are members of the NKRP1 gene family of NK cell receptors located in the immediate vicinity of the Dectin-1 gene cluster (Fig. 2A) (*32*, *33*). All are type II transmembrane proteins and group V CTLRs, which are characterized by having a single extracellular C-type lectin-like domain (CTLD) that lacks the calcium binding site present in the homologous domains of bona fide C-type lectins (*14*, *34*).

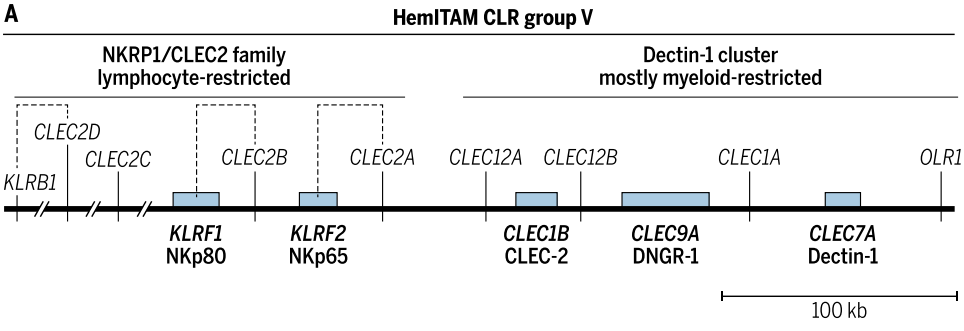
Sequence alignment of these hemITAM-bearing receptors reveals not only the core module YxxL but also an upstream stretch of three negatively charged amino acids, the so-called triacidic motif (Fig. 2B) (*35*–*37*). Thus, a full hemITAM can be defined as the combination of the triacidic motif and the subsequent tyrosine core module. Notably, ITAM sequences also include a highly conserved acidic amino acid three residues upstream of the first tyrosine (Fig. 1) (*6*). The respective functional contributions of these hemITAM features will be discussed below in the context of the prototypical hemITAM receptor CLEC-2. However, first, the cellular distributions and diverse functions of the various hemITAM-bearing receptors are described.

Notably, hemITAMs have only been found thus far in CTLRs, whereas other tyrosine-based motifs are primarily present in immunoreceptors of the IgSF. Hence, one may speculate that the functionally opposing single tyrosine modules ITIM and hemITAM, both of which are found in the cytoplasmic region of CTLRs, may represent evolutionary more ancient tyrosine signaling modules, with the ITAMs, which include two sequential tyrosine core modules, arising later, possibly through duplication of a hemITAM and subsequent spatial segregation to specialized adaptor chains.

HemITAM Receptors

CLEC-2

CLEC-2 (encoded by *CLEC1B*) is a type II transmembrane glycoprotein with a single CTLD (*38*) and reportedly forms non–disulfide-linked homodimers despite the presence of cysteines in the stalk region (*39*). Originally, CLEC-2 was found to act as a receptor of the snake venom rhodocytin (*24*), with the transmembranous glycoprotein podoplanin



B

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CLEC-2      ........MQDEDGYITLNIKTRKPALISVGSASSSWWRVM.....
Dectin-1    MEYHPDLENL-----TQ-HFDSQSNTR-A-V-EKG-CAASP.PWR.
DNGR-1      .........-HE-EI-TS-QWDSPA-DTYQKCLS-NKCSGACCL...
NKp80       ..........----ER-M---VQSK-RSSAQTSQLTFKDYS-TLHWYK
NKp65       .........-EN----M--SF-N-CKSKQKSKDF-LYPQ........

HemITAM consensus        dE[de]gYxxL
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Fig. 2. Genomic organization of hemITAM-utilizing receptors and alignment of hemITAM sequences. (**A**) Genomic organization of a subregion of the natural killer gene complex (NKC) on human chromosome 12. HemITAM-utilizing receptors are indicated as blue bars with labels below the chromosome and grouped in either the NKRP1/CLEC2 family or the Dectin-1 cluster. Intermingled genes are depicted for completeness with labels above the chromosome, and receptor-ligand pairs are denoted by dashed lines. (**B**) Amino acid sequence alignment of the cytoplasmic portions of hemITAM-utilizing receptors. The tyrosine core module is indicated in bold, and the triacidic motif is highlighted in gray. Amino acids identical to the upper CLEC-2 amino acid sequence are shown as dashes, and missing amino acids are depicted by dots. The consensus sequence is shown below, with "x" denoting any amino acid.

later identified as an endogenous cellular ligand of CLEC-2 (*24*, *40*). Podoplanin is expressed on multiple cell types such as lymphatic endothelial cells, type I alveolar cells, and keratinocytes but also on some tumor cells (*41*). In the original report on human CLEC-2 (*38*), the broad presence of transcripts encoding this protein was noted in bone marrow cells and among peripheral blood mononuclear cells, including myeloid cells such as monocytes, DCs, and granulocytes, and a few NK cell clones, indicating a heterogeneous expression pattern. However, CLEC-2 glycoproteins turned out to be mostly restricted to platelets and were also detected at low levels on liver-resident Kupffer cells (*24*, *42*, *43*). In mice, Clec-2 was found not only on platelets but also on neutrophils and activated monocytes (*36*) and, in a subsequent study, also on macrophages, DCs, and a subset of NK cells (*44*). The latter study also reported accumulation of Clec-2 on myeloid cells and NK cells upon lipopolysaccharide (LPS) challenge together with a de novo appearance of Clec-2 on B cells (*44*). A more recent report, however, attributed Clec-2 on mouse B cells to the acquisition from exogenous sources, for example, by trogocytosis, and showed that Clec-2 on mouse myeloid cells is lost during the entrance of these cells into secondary lymphoid organs (*45*). The function of CLEC-2 on platelets is well defined as stimulating platelet activation and aggregation (*10*, *24*, *41*). Chimeric receptors composed of the cytoplasmic tail of Clec-2 fused to the transmembrane and extracellular domains of Dectin-1–stimulated phagocytosis and the production of proinflammatory cytokines by mouse neutrophils, but not a respiratory burst (*36*). Other reports indicated that in DCs, Clec-2 does not trigger cytokine production in the absence of additional triggers. Rather, Clec-2 ligation on myeloid cells was shown to selectively potentiate TLR-mediated interleukin-10 (IL-10) production (*44*). The multiple biological functions of the interaction of CLEC-2 with its natural ligand podoplanin also include a role in blood-lymphatic vessel separation during development, the maintenance of vascular integrity under inflammatory conditions, and wound healing [reviewed in (*41*)].

DECTIN-1

DECTIN-1 (encoded by *CLEC7A* in humans) is a type II transmembrane glycoprotein originally identified in both murine and human DCs (*46*–*48*). Because Dectin-1 lacks cysteine residues in the stalk region, it forms non-disulfide-linked homodimers (*49*). Dectin-1 is a receptor for β-1,3-glucans found in the cell walls of bacteria, fungi, and yeast, and thus is classified as a pattern-recognition receptor (*50*). In addition, there are also reports that Dectin-1 ligates an undefined self-ligand on human T cells thereby providing costimulatory signals (*46*, *51*). In mice, Dectin-1 is found on myeloid cells such as DCs, monocytes, macrophages, and neutrophils (*52*) as well as on a subset of γδ T cells (*53*). The expression profile in humans is even broader, with DECTIN-1 being additionally found on B cells, eosinophils, and mast cells (*54*, *55*). Of note, the abundance and distribution of human DECTIN-1 reportedly is not altered during inflammatory conditions (*54*). In mouse macrophages, Dectin-1 activates not only phagocytosis (*56*, *57*), but also the production of reactive oxygen species (ROS) (*35*). In mouse DCs, Dectin-1 also triggers the production of cytokines such as interleukin-2 (IL-2) and IL-10, thereby promoting differentiation of Th1 and Th17 helper T cells (*23*, *58*). Dectin-1–mediated cytokine production synergizes with TLR2 signals in both mice and humans (*59*–*61*) in a manner that depends on both Syk kinase and MyD88 (*62*). Dectin-1 ligation on DCs was further shown to capacitate DCs for priming of cytotoxic $CD8^+$ T cells (*63*). Dectin-1–activated cross-priming of cytotoxic $CD8^+$ T cells by DCs depends on caspase recruitment domain family member 9 (Card9) but does not depend on the activation of inflammasomes (*64*). In humans, DECTIN-1 stimulates mast cells to produce leukotrienes (*55*), which have recently been shown to be involved in the activation of ILCs (*65*), thereby providing another line of cross-talk between myeloid cells and innate lymphocytes. In addition, DECTIN-1 may contribute to wound healing because DECTIN-1 activation by β-glucans induces proliferation and migration of human keratinocytes (*66*). Recently, two isoforms of DECTIN-1 in human monocytes were described as differing in the presence (isoform A) or absence (isoform B) of the stalk region. Upon maturation of monocytes, the abundance of isoform B increased, whereas the abundance of isoform A decreased. Both isoforms seem to differ with regard to their subcellular localization and cytokine secretion profile (*67*).

DNGR-1

DNGR-1 (encoded by *CLEC9A*) is a type II transmembrane CTLR that exists as a disulfide-linked homodimer primarily on human $BDCA3^+$ DCs and mouse $CD8\alpha^+$ DCs but also on mouse plasmacytoid DCs (*68*–*70*), enabling these antigen-presenting cells to sense exposed F-actin as a damage-associated molecular pattern (DAMP) of

necrotic cells (*25*). DNGR-1 binds to actin filaments of damaged or dying cells (*71*–*73*), thereby promoting endocytosis of dead cell–associated antigens and presentation of these antigens on MHC class II as well as their cross-presentation on MHC class I. Such DNGR-1–triggered antigen presentation by DCs has been reported to allow for the generation of adaptive immune responses to these antigens without activating DCs (*25*, *69*, *70*, *74*, *75*).

NKp80

NKp80 (encoded by *KLRF1*) is a type II transmembrane CTLR expressed as a disulfide-linked homodimer on human peripheral blood NK cells (*76*) and a subset of effector memory T cells (*77*). NKp80 binds to the C-type lectin-like glycoprotein AICL that is encoded adjacently to the *KLRF1* locus in the NKC and present on myeloid cells (*78*) and monokine-experienced NK cells (*79*). An NK-monocyte cross-talk leads to mutual activation of NK and myeloid cells, resulting in cytokine production (*78*), whereas AICL-positive NK cells are rendered susceptible to cytotoxicity through bystander NK cells, which are in turn activated to produce cytokines (*79*). Further, NKp80 has been identified as a marker for mature cytotoxic, perforin-expressing NK cells in NK cell development (*80*).

NKp65

NKp65 (encoded by *KLRF2*) is a type II transmembrane CTLR and a non–disulfide-linked homodimer (*28*, *29*). NKp65 is present on the human NK lymphoma cell line NK-92 but not on peripheral blood NK cells. The cellular distribution of NKp65 receptors in vivo remains to be shown. NKp65 tightly binds to the C-type lectin-like glycoprotein KACL, which is encoded adjacently to *KLRF2* in the NKC and restricted to keratinocytes (*28*, *81*). The exceptionally high affinity of the NKp65-KACL interaction (*28*, *82*) is due to high shape complementary and a few "hotspot" amino acid residues, providing a high number of hydrogen bonds (*29*, *82*). NKp65 triggering stimulates calcium flux, degranulation, and cytotoxicity of NK-92 cells in a Syk-dependent manner (*28*, *29*).

Summary

Receptors that use hemITAMs can be roughly categorized into those that are primarily present on myeloid cells and those that are present exclusively on lymphoid cells. The natural ligands of these hemITAM-coupled receptors are heterogeneous and include both pathogen-associated molecular patterns (PAMPs) and DAMPs, such as β-glucans (Dectin-1) and F-actin (DNGR-1), respectively, but also the snake venom protein rhodocytin (CLEC-2) and endogenous molecules such as podoplanin (CLEC-2), KACL (NKp65), and AICL (NKp80) (Table 1). In myeloid cells, hemITAM receptors stimulate phagocytosis and cytokine production, but in lymphoid cells, they stimulate the cytotoxic program and cytokine production. Even within the same cell lineage, there are important functional differences between these hemITAM receptors. DNGR-1 was shown to stimulate only endocytosis and cross-presentation but not to activate DCs (*25*, *69*, *70*, *74*). This is in contrast to Dectin-1, which not only stimulates endocytosis and cytokine production but also activates DCs (*63*). Further, the cytoplasmic tail of Dectin-1, but not that of CLEC-2, stimulates a respiratory burst in mouse macrophages, although both receptors signal through Syk, which promotes ROS production (*35*, *36*). In NK cells, NKp80 primarily triggers cytotoxicity, but a subtle alteration of the hemITAM results in a strongly enhanced cytotoxicity and cytokine production (*26*, *27*). Collectively, hemITAM receptors are capable of triggering effector functions in both myeloid

Table 1. Natural ligands, expression profiles, and functions of hemITAM receptors. HemITAM receptors are C-type lectin-like receptors (CTLRs) that are encoded by genes clustered together in the natural killer gene complex (NKC), which includes the Dectin-1 cluster and members of the NKRP1 receptor family. The expression profile for each receptor is indicated for both mice (m) and humans (h). DC, dendritic cell; ROS, reactive oxygen species; T_{EM} cells, effector memory T cells; TLR, Toll-like receptor.

Receptor	Ligand	Expression	Function
Dectin-1 cluster			
CLEC-2	Rhodocytin Podoplanin	m: platelets, neutrophils, monocytes, macrophages, DC, NK h: platelets, liver-resident Kupffer cells	Platelet aggregation Modulation of TLR signaling
Dectin-1	β-1,3-Glucan Undefined cellular ligand	m: DC, monocytes, macrophages, neutrophils, γδ T cells h: DC, monocytes, macrophages, neutrophils, γδ T cells, B cells, eosinophils, mast cells	Phagocytosis ROS production Cytokine production in synergy with TLR2 DC adaptation for priming of CD8 T cells Leukotriene production
DNGR-1	F-actin	m: $CD8\alpha^+$ DC, plasmacytoid DC h: $BDCA3^+$ DC	Cross-presentation of dead cell–associated antigens
NKRP1 receptors			
NKp80	AICL	m: absent in rodents h: NK cells, T_{EM} cells	Cytotoxicity Cytokine production
NKp65	KACL	m: absent in rodents h: NK-92	Cytotoxicity Cytokine production

cells and innate lymphocytes, but these functions vary substantially not only with regard to the cellular context but also between the different hemITAM receptors, depending on the individual hemITAM.

Key Features of HemITAM Signaling as Defined by the Prototypic HemITAM Receptor CLEC-2

HemITAMs are tyrosine-phosphorylated by SFK, Syk, or both

The characteristics of hemITAM signaling have been most studied in CLEC-2 glycoproteins. The hemITAM-embedded tyrosine is critically important not only for CLEC-2 signaling (*24*) but also for signal transduction by Dectin-1 (*23*, *35*), DNGR-1 (*25*), NKp80 (*26*), and NKp65 (*28*, *29*). As originally established for the antigen receptors of T and B cells, immunoreceptor signaling involves the phosphorylation of tyrosine residues by SFKs. Both SFK and Syk inhibitors prevented the tyrosine phosphorylation of mouse Clec-2 when Clec-2 was activated by antibody-mediated cross-linking (*83*). Unexpectedly, however, when mouse Clec-2 was bound to rhodocytin, its phosphorylation depended only on Syk kinase activity but not on SFKs (*83*). In contrast, human CLEC-2 tyrosine phosphorylation upon treatment with rhodocytin was shown to be sensitive to inhibition of both SFKs and Syk kinase (*24*, *37*). Although Syk phosphorylation itself is only moderately sensitive to inhibition of Syk kinase activity, it is entirely dependent on SFK activity (*37*). Hence, it was suggested that an initial round of SFK activity phosphorylates the hemITAM and Syk, leading to Syk activation that in turn mediates further hemITAM phosphorylation and Syk autophosphorylation (*37*). Thus, CLEC-2 seems to be phosphorylated differently in mice depending on whether it is activated by rhodocytin or antibody cross-linking, and it is phosphorylated differently in mice and humans in response to rhodocytin.

Binding to its natural ligand zymosan, a component of fungal cell walls, stimulates tyrosine phosphorylation of mouse Dectin-1 (*60*). Whether this induces phagocytosis depends either on SFK and Syk kinase activity, as shown in a mouse fibroblast cell line, or only on SFK activity in macrophages (*57*), revealing differences of the proximal signaling mechanism based on the cellular context. Data on the involvement of Syk in DNGR-1–mediated endocytosis are scarce. Using chimeras of the cytoplasmic domain of human DNGR-1 fused to the transmembrane and extracellular domain of mouse Dectin-1 transfected into Syk-deficient versus Syk-sufficient mouse B cell lines, it was concluded that DNGR-1 signals through Syk kinase (*68*). In addition, Dngr-1 on mouse DCs triggers Syk phosphorylation in response to conjugation with dead cells and promotes the cross-presentation of dead cell–associated antigens. Cross-presentation, but not uptake, of antigens was blocked in Syk-deficient DCs, indicating that a Dngr-1–Syk pathway supports cross-presentation (*25*).

NKp80 also becomes phosphorylated at the hemITAM-embedded tyrosine upon treatment with the tyrosine phosphatase inhibitor pervanadate (*26*). This depends on SFKs, but not on Syk kinase activity, in NK-92MI cells (*27*), whereas NKp80-triggered cytotoxicity depends on both SFKs and Syk kinase (*26*). NKp65 was recently shown to become phosphorylated at the hemITAM-embedded tyrosine in NK-92MI cells upon antibody ligation or binding to its endogenous ligand KACL, with NKp65-stimulated degranulation depending on Syk kinase activity (*29*).

The involvement of the Syk kinase family member ζ chain–associated protein kinase 70 (ZAP-70) in CLEC-2 signaling was assessed using Syk-deficient chicken DT40 cells and ZAP-70–deficient human Jurkat cell lines, in which either Syk or ZAP-70 expression was reconstituted transgenically. These experiments showed that only expression of Syk, but not expression of ZAP-70, allowed CLEC-2 signaling, indicating that only Syk can be utilized by CLEC-2 for signal transduction (*37*). This same Syk exclusivity was shown for NKp80 signaling: Overexpression of a kinase-dead form of Syk, but not a kinase-dead form of ZAP-70, blocked NKp80-triggered cytotoxicity (*26*).

CLEC-2 tyrosine phosphorylation and subsequent platelet aggregation were originally reported to require translocation of CLEC-2 into lipid rafts upon ligand engagement (*84*). However, a later study showed that neither CLEC-2 phosphorylation nor subsequent Syk activation depends on lipid raft integrity, leading to the proposal that lipid raft disruption inhibits the actions of secondary mediators necessary for CLEC-2–triggered platelet aggregation rather than directly interfering with CLEC-2 phosphorylation (*85*). In addition, CLEC-2 phosphorylation and activation of Syk were also inhibited when actin polymerization was blocked by cytochalasin D, whereas Syk activation stimulated by the ITAM-coupled GPVI receptor was unaffected (*84*). It remains to be shown whether membrane localization and actin polymerization also affect the function of other hemITAM receptors.

HemITAM and Syk—Do they couple?

A central principle of ITAM-based signaling by activating antigen receptors involves the recruitment of Syk kinase family members, Syk or ZAP-70, to the tyrosine-phosphorylated ITAM. Syk recruitment by hemITAM receptors has been almost exclusively studied using reductionistic approaches, such as pulldown experiments using peptides encompassing the hemITAM or the recombinantly produced tandem SH2 (tSH2) domains of Syk (Syk tSH2). For example, CLEC-2 was precipitated from human platelets using Syk tSH2, and similarly, Syk was precipitated from human platelets using CLEC-2 hemITAM peptides, suggesting a direct recruitment of Syk to the tyrosine-phosphorylated hemITAM of CLEC-2 (*10*, *24*). Further experiments led to the hypothesis that Syk cross-links two CLEC-2 monomers through the interaction of the SH2 domains of Syk with the hemITAM tyrosine modules of two CLEC-2 molecules, thereby forming an ITAM-like complex (*86*). Notably, Syk tSH2 binding to ITAM peptides was about ~10-fold stronger than to hemITAM peptides, indicating that there is a higher affinity for two tyrosine motifs on the same polypeptide compared to two single tyrosine motifs on separate polypeptides (*86*). With regard to Dectin-1 and DNGR-1, the evidence for Syk recruitment by the tyrosine-phosphorylated hemITAM is scarce and primarily relies on the pulldown of Syk by peptides encompassing the respective tyrosine-phosphorylated hemITAM sequences (*23*, *68*). However, a direct interaction with Syk has not been demonstrated for any of these hemITAM receptors by coimmunoprecipitation in a physiological setting.

Initial studies on NKp80 using NKp80 hemITAM peptides suggested Syk recruitment to the phosphorylated hemITAM (*26*). However, such an association could not be confirmed for full-length NKp80 glycoproteins (*27*) or Syk tSH2-GST fusion proteins (*29*). Similarly, cellular Syk could not be coimmunoprecipitated with intact CLEC-2, and vice versa, from lysates of stimulated platelets (*24*). Mutational analyses of the atypical hemITAM of NKp80 revealed that reconstitution of the hemITAM consensus sequence was sufficient to allow efficient Syk recruitment to this hemITAM-optimized NKp80 (NKp80-DG). NKp80-DG, with Glu^5 mutated to Asp (D) and Arg^6 mutated to Gly (G), stimulates a substantially stronger

degranulation and cytotoxicity as compared to wild-type NKp80 (*27*). Whereas Dectin-1 and CLEC-2 both use a consensus hemITAM, the hemITAM of DNGR-1 also deviates from the consensus at position 6, where the consensus glycine is replaced by an isoleucine. Accordingly, another NKp80 mutant (NKp80-EIY) where Arg^6 was replaced by Ile, as in the hemITAM of DNGR-1, also failed to recruit Syk kinase and was functionally attenuated similarly to wild-type NKp80 (*27*). DNGR-1 mediates endocytosis and cross-presentation but, unlike Dectin-1, does not promote DC activation (*25*, *63*, *69*, *70*, *74*), which points toward a profound difference in signaling quality. It is tempting to speculate that the glycine to isoleucine substitution adjacent to the phosphotyrosine critically affects Syk recruitment also in DNGR-1 and thereby contributes to the observed differences in functionality. NKp80 is the only hemITAM-bearing receptor in NK cells, but defining its specific contribution to NK cell function will have to wait until the SH2 domain–containing proteins that are recruited to the tyrosine-phosphorylated hemITAM of this receptor are identified. Last, NKp65 does not recruit Syk as evident from both Syk-tSH2-GST fusion protein pulldown experiments and coimmunoprecipitation studies regardless of prior antibody- or ligand-mediated receptor stimulation (*29*).

Most hemITAMs contain a stretch of three negatively charged amino acids, termed the triacidic motif, upstream of the tyrosine module (Fig. 2B). Mutational studies with CLEC-2 revealed that the triacidic motif critically determines the extent of tyrosine phosphorylation and the binding of Syk tSH2 to the hemITAM (*37*). An ITAM receptor in which one of the two tyrosine residues was mutated to phenylalanine became functionally silent, whereas introduction of the triacidic motif upstream of the remaining tyrosine restored signaling (*37*). Hence, the triacidic motif is particularly relevant for hemITAM function. In NKp80, the function of the triacidic motif may be modulated by the positively charged Arg^6, because mutating this Arg to the consensus glycine led to a strongly increased tyrosine phosphorylation (*27*). In NKp65, the triacidic motif is interrupted by the presence of the polar amino acid Asn, and mutational reconstitution of the triacidic motif by replacing this Asn by Asp increased tyrosine phosphorylation of this motif (*29*). However, in both cases, increased tyrosine phosphorylation did not result in enhanced functionality in terms of cytotoxicity (*27*, *29*). This triacidic motif upstream of the hemITAM-embedded tyrosine creates an optimal site for phosphorylation by SFKs and for the binding to Src kinase SH2 domains (*87*). Consequently, the primary function of the hemITAM may be to activate SFKs, which subsequently activate Syk.

Syk Activation Without Recruitment by a HemITAM

Syk is a nonreceptor tyrosine kinase belonging to the Syk kinase family, which also includes ZAP-70. Syk comprises two SH2 domains and a kinase domain linked through two interdomains, resulting in a high structural flexibility and allowing the two SH2 domains to interact with the two tyrosine core modules in ITAMs (*88*–*90*). In the resting

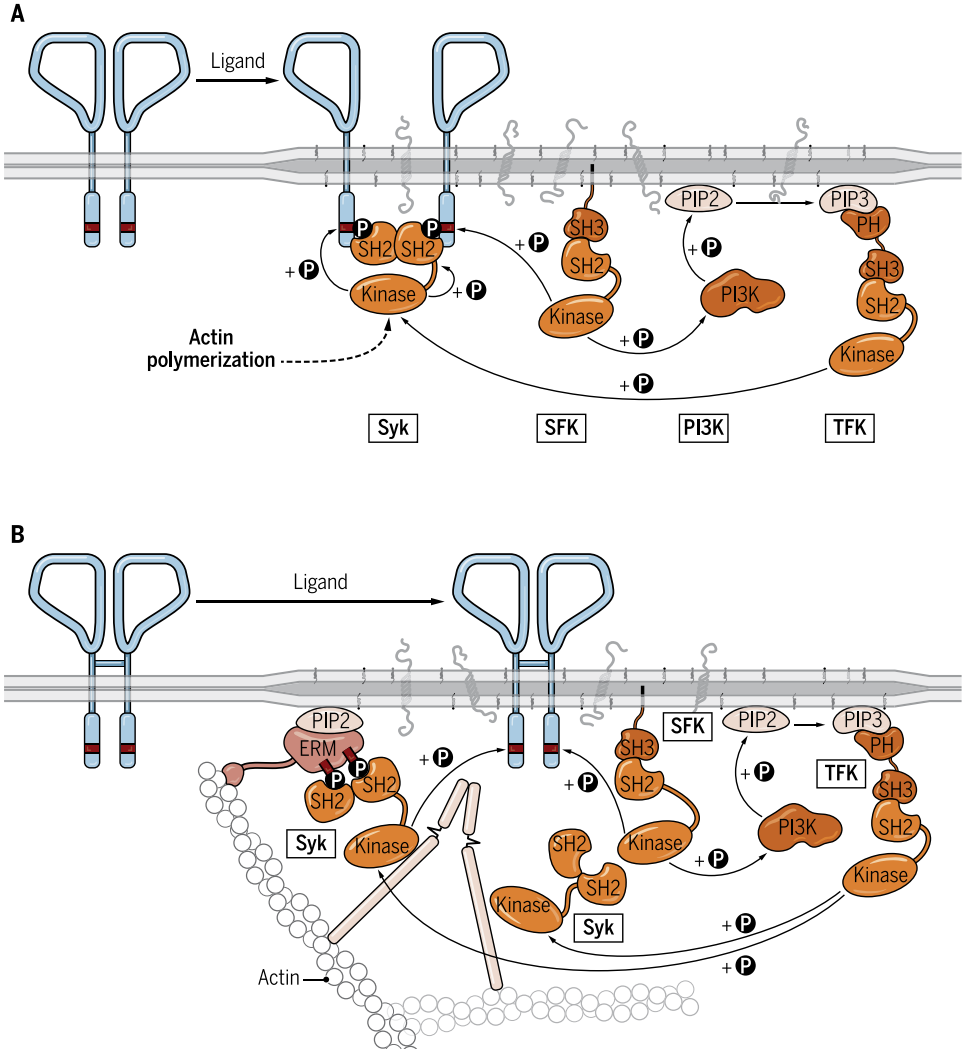

Fig. 3. Models for proximal signaling events initiated by hemITAM receptors. (**A**) Current model of CLEC-2 signaling in which tyrosine phosphorylation and Syk phosphorylation depend on relocalization of CLEC-2 into lipid rafts and actin polymerization. The hemITAM is initially phosphorylated by Src family kinases (SFKs), which also activate phosphatidylinositol 3-kinase (PI3K), leading to the production of phosphatidylinositol 3,4,5-trisphosphate (PIP_3) from phosphatidylinositol 4,5-bisphosphate (PIP_2). PIP_3 creates docking sites for Tec family kinases (TFKs), which in turn become activated and phosphorylate Syk. Syk further phosphorylates and associates with the hemITAM. (**B**) Alternative model of the hemITAM proximal signaling mechanism as deduced from several studies (*94*–*96*). Relocalization of Syk to the plasma membrane upon actin polymerization is facilitated by filamin A or ITAM-containing ezrin/radixin/moesin (ERM) proteins, or both, in the absence of a direct association of Syk with the hemITAM-containing immunoreceptor.

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state, the kinase domain of Syk is autoinhibited; it is activated either by binding of the two SH2 domains to an ITAM or through tyrosine phosphorylation by SFK or by autophosphorylation. Syk activation is therefore considered an OR switch (*91*). In contrast, ZAP-70 lacks such interdomain flexibility, making it more structurally restrained, and is also less readily autophosphorylated than Syk (*90*–*92*).

In immunoreceptor signaling, Syk is commonly recruited to the phosphorylated ITAM in response to receptor engagement and activated through phosphorylation by an SFK. However, studies have also identified Syk activation mechanisms that do not depend on Syk binding to ITAM-containing adaptor chains. For example, the P-selectin glycoprotein ligand 1 (PSGL1) stimulates Syk activation. Whereas one study claimed the involvement of adaptor chains DAP12 and FcRγ (*93*), another study proposed an involvement of members of the ezrin/radixin/moesin (ERM) family, which are known for their role in linking the cytoskeleton to the plasma membrane (*94*). In this latter proposed mechanism, ERM proteins bind to PSGL1 and recruit Syk through an ITAM (*94*). In platelets, Syk was found to associate with the actin filament cross-linking protein filamin A, thereby relocalizing Syk to the plasma membrane and rendering it susceptible to activation through tyrosine kinases (*95*). In a more recent study with CLEC-2, it was shown that Syk activation depends on the Src-PI3K-Tec pathway (*96*). Although it was concluded that Tec family kinases (TFKs) phosphorylate Syk in a CLEC-2–bound state, the requirement for a CLEC-2–Syk association was not shown (*96*). Hence, this Syk activation pathway may possibly represent a mechanism of Syk activation that is independent of Syk recruitment to a hemITAM. It remains to be determined whether the Src-PI3K-Tec-Syk pathway also operates downstream of other hemITAM-coupled receptors. In this model, a direct interaction of Syk with the hemITAM would not be required because Syk is capable of being relocalized to the plasma membrane and activated by the actions of ERM, filamin A, or the Src-PI3K-Tec pathway.

Proposal of an Alternative HemITAM Signaling Mechanism

The signaling mechanism of CLEC-2 was proposed to involve an initial round of tyrosine phosphorylation of the hemITAM and Syk by SFK, leading to Syk recruitment, Syk activation, Syk autophosphorylation, and further phosphorylation of the hemITAM by Syk (*37*). A prerequisite for signaling initiation may be the relocalization of CLEC-2 into lipid rafts and polymerization of the actin cytoskeleton. It has also been reported that Syk activation by SFK is mediated through PI3K and TFK activity (Fig. 3A) (*96*).

Other findings concerning Syk activation as described above do not necessarily depend on Syk recruitment to the hemITAM. In such a scenario (Fig. 3B), Syk would be recruited to the plasma membrane either by associating with filamin A or by binding to ITAMs in ERM proteins that are associated with the plasma membrane or with membrane receptors. Hence, two pools of Syk proteins would be present in the vicinity of the active SFKs that phosphorylate and activate Syk. Such a mechanism might explain the dependency of CLEC-2–stimulated Syk phosphorylation on actin polymerization. The role for the hemITAM-embedded tyrosine in this scenario remains unresolved and may serve merely as a docking site for other SH2 domain–containing signaling proteins or for SFK, thereby enriching SFK kinases at the site of activation. It is important to note that both models are not mutually exclusive and that the alternative pathway might compensate for the low affinity of atypical hemITAMs for Syk, thus augmenting Syk activation in the context of hemITAM-dependent signaling.

Conclusions

Activating immunoreceptors containing cytoplasmic hemITAMs are present on various myeloid cells and innate lymphocytes and control different cellular processes such as phagocytosis, cytokine production, and cytotoxicity depending on both the ligand that initiates signaling, and the cellular context. The signaling mechanisms upon ligation of these hemITAM-utilizing receptors are diverse; they differ not only between receptors but also between different cell types. These differences are likely due to amino acid alterations in the hemITAM sequence and other structural constraints of the respective hemITAM immunoreceptor. Specifically, although Syk is involved in signaling downstream of receptors that use hemITAMs, the recruitment of Syk to the hemITAM appears to vary between different hemITAM-bearing receptors, although the involvement of Syk in the downstream signaling pathways is required. For example, NKp80 and NKp65 apparently do not recruit Syk upon receptor stimulation, and therefore, one may reconsider the term hemITAM to differentiate such motifs from Syk-recruiting ITAMs. More studies on the sequence of signaling events upon ligation of hemITAM-utilizing receptors are needed to clarify the underlying signaling pathways that mediate receptor function.

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Funding: Our work on NKp65 and NKp80 signaling mechanisms is supported by the Deutsche Forschungsgemeinschaft (grants STE 828/5-2 and STE 828/6-2 to A.S.).
Competing interests: A.S. holds patents on NKp65 and NKp80. B.B. declares no conflict of interest.

Submitted 4 April 2017
Accepted 16 October 2017
Published 5 December 2017
10.1126/scisignal.aan3676

Citation: B. Bauer, A. Steinle, HemITAM: A single tyrosine motif that packs a punch. *Sci. Signal.* **10**, eaan3676 (2017).

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Sci. Signal. **10** (508), eaan3676.
DOI: 10.1126/scisignal.aan3676