Receptor mediated inhibition in regulation of NK cell function

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ABSTRACT

Natural Killer cells are capable of directly recognizing pathogens, pathogen-infected cells as well as transformed cells. NK cells recognize target cells using approximately 100 germ-line encoded receptors, which display activating or inhibitory function. NK cell activation usually requires the engagement of more than one receptor and these contribute in part distinct signaling inputs that are required for the firm adhesion of NK cells to target cells, the polarization and the release of cytotoxic granules and the production of cytokines. Here we will discuss how receptor-mediated intracellular inhibitory signaling occurs and how it can dominantly interfere NK cell activation signaling events. In addition we will discuss receptors that can interfere with cellular activation already at the level of receptor-ligand interactions. Such receptor-mediated inhibitions of NK cell function serve three main purposes: Ensuring tolerance to normal cells and enabling NK cell responses to aberrant host cells that have lost inhibitory ligand and finally allowing the recognition of certain pathogens that do not express inhibitory ligands.

INTRODUCTION

NK cells are a lymphocyte subpopulation capable of directly recognizing pathogens (1) as well as pathogen-infected cells (2). In addition NK cells have long been known to recognize transformed cells (3) and they are increasingly recognized for their immune regulatory roles (4). They can recognize their targets independent of somatically rearranged antigen-receptors and use up to 100 germ-line encoded NK cell receptors, which display activating or inhibitory function (for review see (5, 6)). While activating receptors are usually expressed on all NK cells, inhibitory receptors often show restricted expression, which creates significant NK cell diversity. In addition to the invariant target recognition structures, NK cells acquire an effector cell program already in their tissue of origin. Bone marrow NK cells constitutively express high levels of Perforin, Granzyme B and IFNγ mRNAs that are, however, not translated into protein (7, 8). The trigger for the acquisition of this effector program is not known, but IL-17 signaling may play a role (9).

Subsequent to these developmentally programmed events, NK cells undergo significant phenotypic changes based on environmental cues. They adapt their responsiveness to the inherited MHC class I molecules (10) and undergo further maturation (CD11b, CD27, KLRG1) in response to the presence of other cell types (11). Finally, NK cells are critically impacted by "priming" i.e. their exposure to inflammation induced cytokines, such as IL-15 in the case of viral (12) or IL-12 during bacterial infection (13). Priming allows the translation of preformed Perforin and Granzyme B mRNA, enhances IFNγ production, can induce the expression of additional NK cells receptors (NKp44) and render signaling by activation receptors more effective (14). The exocytosis of lytic granules and the production and release of cytokines upon interaction with a target cell is then tightly controlled by signals

transduced via inhibitory and activating NK cell receptors that interact with ligands on the surface of target cells. The generally accepted view holds that an excess of activating over inhibition signals results in an NK cell effector response, which includes the release of lytic granules containing Perforin/Granzyme (to mediate cytotoxicity) and the release of inflammatory cytokines such as IFNγ and TNFα. A final adaptive process based on the stimulation of NK cells (e.g. by viral ligand such as MCMV m157) is the acquisition of memory-like features, which include increased longevity and improved effector capacities of NK cells (15).

1. NK CELL ACTIVATING RECEPTORS AND SIGNALING

In order to understand the importance and the mode of action of inhibitory receptors, we will first briefly summarize the various signaling pathways implicated in NK cell activation.

Receptors associated with membrane bound signaling adaptors

Similar to antigen receptors, multiple activation receptors expressed by NK cells depend on non-covalently associated, membrane anchored adaptors for signal transduction. Some of these adaptors (DAP12, FcRγ □□□□CD3ζ) contain one or three ITAMs (Immunoreceptor Tyrosine-based Activating Motif). NK cell receptors associated with DAP12 include human NKp44 and mouse NKG2D. Those associated with FcRγ include mouse CD16 and NK1.1, while CD3ζ □□□□FcRγ □□□□□□□□□□are associated with human CD16 or human NKp30. Receptor engagement activates Src family tyrosine kinases (including Lck and Fyn), which phosphorylate ITAMs in NK cells and allow the recruitment of the non-receptor tyrosine kinases Syk and Zap70. Subsequently, both trans membrane (Linker

of activated T cells (LAT) and Non-T cell activation linker (NTAL)) and cytosolic adaptors (SLP-76 and Vav2/ 3) are activated, leading to the activation of PI3K, ERK and PLC-γ1/2 (6).

In addition to ITAM bearing adaptors, several activation receptors (NKG2D, Ly49H) also associate with DAP10, a homodimeric membrane-anchored signaling adaptor with an YxxM motif (in single-letter amino acid code, with x indicating non-conserved positions). The same motif is present in receptors that co-stimulate T cells, such CD28 or ICOS. Phosphorylated DAP10 recruits the p85 subunit of phosphoinositide 3-kinase (PI3K) and the small adaptor Grb2 in association with Vav1.

NK cell activation independent of membrane bound signaling adaptors

NK cells express additional activating cell surface receptors that function independent of an association with membrane-anchored adaptors. SLAM (signaling lymphocytic activation molecule) family receptors activate NK cells using semi-conserved S/T/I/VYxxV/I motifs (termed ITSM for Immune receptor Tyrosine based Switch Motifs), which are present in the cytoplasmic portion of multiple receptors. The best understood receptor is 2B4 (CD244). Upon ligand binding and phosphorylation, the ITSM associates with small cytoplasmic adaptors such as signaling lymphocytic activation-associated protein (SAP) (also termed SH2D1A), EAT-2 and ERT. SAP mediates activating function by coupling SLAM family receptors to the Src tyrosine kinase Fyn, which links SAP to the exchange factor Vav-1. SAP-Fyn contributes to the formation of conjugates between NK cells and target cells (16). EAT-2 does not promote conjugate formation but accelerates polarization and exocytosis of cytotoxic granules (17), providing an explanation why both adaptors are needed for normal NK

cell effector function. However, as detailed below, in certain cases SLAMs can act as inhibitory receptors.

Additional receptors, including CD44, CD137, the TNF receptor family member CD27, CD160 and DNAM-1 contribute to NK cell activation signaling. However, the membrane proximal signaling events induced by these receptors are less well understood.

Leucocyte Function-associated Antigen-1 (LFA-1)

The β2 integrin LFA-1 (CD11a/CD18) plays a key role for NK cell function by mediating adhesion to target cells. To do so, LFA-1 must first undergo a conformational change to achieve a high-affinity binding state. LFA-1 activation in NK cells occurs by inside out signals from co-activating receptors such as NKG2D, DNAM-1 or 2B4 (18), allowing firm adhesion.

In addition, in NK cells LFA-1 has the ability to signal on its own and to function as a co-activating receptor. The engagement of LFA-1 by its ligand ICAM-1 on target cells is sufficient to polarize (but not release) lytic granules in human NK cells (19). Ligation of LFA-1 leads to phosphorylation and activation of Vav1 and the kinase Pyk2, which together with the Wiskott-Aldrich syndrom protein (WASp) (20) regulate actin polymerization. While binding of ICAM-1-coated beads to mouse NK cells is also sufficient to induce reorganization of the actin cytoskeleton, lytic granules do not polarize (21). Thus increase adhesive function of LFA-1 is an additional key feature common to productive NK cell activation.

Redundancy and selectivity of NK cell activation signaling

NK cell receptors have generally been classified into activation and co-activation receptors depending on whether an individual receptor is able to trigger an effector response or whether receptor co-engagement is required. Based on NK cell stimulations using natural ligands, only CD16 qualified as an activation receptor in non-activated human NK cells. For all other receptors, pairwise engagement was needed to induce target cell lysis by resting human NK cells (22). A common consequence of the engagement of activating NK cell receptors is the phosphorylation of Vav family members. ITAM containing adaptors activate Vav-2 and 3, while the YxxM containing adaptor DAP10 (23) and switch motifs activate Vav-1 (16). A certain threshold level of activation seems to be required to overcome an inhibitory effect of c-Cbl on Vav-1 phosphorylation (24). The basis for synergy is that co-engagement of receptors (e.g. human 2B4 and NKG2D) phosphorylates two distinct tyrosine residues (Y113 and Y128) in the adaptor protein SLP76. While receptor combinations that do not synergize phosphorylated either one of the two tyrosines (25). Thus SLP76-mediated integrates signals from distinct (co-) activation receptors and phosphorylates at two distinct tyrosine residues is essential to overcome the inhibitory effect of c-Cbl and on Vav-1 activation (24).

A corresponding analysis of murine NK cells is currently lacking. Based on mAb stimulation, several receptors, including NK1.1, NKG2D, NKp46, Ly49H and CD16 can be considered activating in NK cells from naive mice (10). Species-specific differences in adaptor usage and/or signal transduction may explain some of these dissimilarities. Irrespectively, it is worth noting that need for co-engagement depends on whether and to what extent NK cells have been exposed to cytokines in vivo or *in vitro* (14).

2. Receptor mediated inhibition of NK cell function

Inhibitory receptors specific for MHC class I molecules

The existence of inhibitory receptors was predicted based on negative effects of MHC class I molecules expressed by target cells on NK cell effector responses. Inhibitory receptors specific for classical MHC class I molecules expressed by human NK cells belong to the Killer Immunoglobulin-like Receptor (KIR) family. In addition, human NK cells express Leukocyte Immunoglobulin-like receptor B2 (LILRB1 also termed LIR-1), a pan MHC class I receptor and CD94/NKG2A a receptor for non-classical HLA-E. Mouse NK cells also express CD94/NKG2A, a receptor for Qa-1b (the orthologue of human HLA-E). In addition they express Ly49 family receptors for the detection of classical and certain non-classical MHC class I molecules, such as H2-M3 (26). In addition to these MHC class I receptors, NK cells express a significant number of inhibitory receptors, which are not specific for MHC class I ligands, such as Siglecs (Sialic acid binding Ig-like lectins), LAIR1 and NKR-P1A. In general, these receptors prevent responses to "normal-self" cells and allow responses to "missing-self" cells that lack inhibitory ligand.

A standard model of receptor mediated inhibition

Inhibitory receptors block NK cell function by interfering with activation signals. They act locally and block only signaling by activation receptors that are co-aggregated. An illustration of this notion is that, the integration of activating and inhibitory receptor signals depends on the receptor-ligand complexes to have similar dimensions. Indeed, activation and inhibitory ligand pairs co-localize when their respective sizes match, whereas they are segregated when their sizes differ (27, 28). Finally, despite the diversity of the early activating signaling pathways, inhibitory receptors can generally

counteract NK cell activation.

Typical receptors with inhibitory function are characterized by the presence of conserved I/VxYxxL/V motifs (Immunoreceptor Tyrosine-based Inhibitor Motif (ITIM)) in the cytoplasmic portion of the receptor. Each receptor complex contains two ITIMs e.g. as a tandem arrangement in monomeric KIRs or single ITIMs per subunit in homodimeric Ly49 receptors. Receptor engagement leads to ITIM phosphorylation, likely by the activity of Src family kinases, including Lyn or Lck (29). Phosphorylated ITIMs recruit and activate the tandem SH2 protein phosphatases SHP-1 and SHP-2, whereby SHP-1 plays an essential role for the inhibitory function of MHC class I receptors. A key substrate of SHP-1 downstream of inhibitory KIR is Vav-1 (30) (**Fig.1A**). As discussed above Vav-1 is phosphorylated by NK cell activation and Vav-1 dephosphorylation prevented the formation of downstream c-Cbl, Crk, p130CAS, C3G complexes (31), which have essential roles for inducing cell adhesion and the activation of the MAP kinase pathway. Dephosphorylation of all Vav proteins by ITIM-SHP-1 would explain a block of most, if not all, NK cell activation pathways. However this remains to be shown.

Available evidence suggests that inhibitory signals block activation upstream of LFA-1 function and upstream of actin-dependent signals for receptor recruitment and phosphorylation. As pointed out above, signals delivered by co-activation receptors can improve LFA-1-dependent adhesion of resting NK cells to target cells (16). Such inside-out signals are blocked when the inhibitory CD94-NKG2A receptor is co-engaged (32). In addition, imaging shows that inhibitory MHC class I receptors rapidly form microclusters upon engagement and that this prevents microclustering of activating receptors (33). In addition, inhibitory signaling efficiently prevents the polarization of lytic granules (34). Thus, rather than allowing activation signals to be

fully established before acting on them, inhibitory receptors prevent activation signals from being propagated.

An alternative mechanism of ITIM-dependent inhibition

The analysis of biochemical events following target cell encounter unexpectedly showed that inhibitory receptor engagement induced unique events, which were not detected when only activation signaling was present. KIR or CD94/NKG2A engagement activated the tyrosine kinase c-Abl, which phosphorylated Crk at Tyr 221 (31). Further experiments showed that phosphorylated Crk is present at inhibitory, but not activating synapses, and that Crk phosphorylation is needed to inhibit NK cells (**Fig. 1B**). This has been explained by a role for unphosphorylated Crk for the clustering and the signaling by activation NK cell receptors such as CD16 (35). Counter to a need for co-aggregation with activation receptors, engagement of CD94-NKG2A by HLA-E alone was sufficient to induce Crk phosphorylation (35), suggesting that at least CD94/NKG2A receptor complexes can exert autonomous signaling function.

Inhibition via immune receptor tyrosine based switch motifs (ITSM)

The description of a consensus ITIM led to the identification of additional receptor families that were correctly predicted to mediate inhibitory function. Many of these new receptors were specific for non-MHC class I molecules. In addition, inhibitory receptors were identified that did not have a consensus ITIM. Selected examples of such receptors and their mode of action are discussed below. Even though SLAM family receptors, including 2B4, have been introduced as activating receptors, under certain conditions these receptors can exert inhibitory roles. For example, in NK cells from patients with X-linked lympho proliferative (XLP) disease 2B4 functions as an

inhibitory receptor. XLP patients have inactivating mutations in the SAP-encoding gene *SH2D1A* gene (36). In the absence of SAP, SLAM receptors interact with SHP-1, SHP-2, Csk and SHIP-1 (SH2 domain containing inositol phosphatase 1). Functional experiments using a B cell line lacking SAP, showed that only SHIP-1 mediated 2B4-dependent inhibition of BCR signaling (16) (**Fig. 1C**). While 2B4 engagement induced SHIP-1 phosphorylation, it is not yet clear how 2B4 recruits SHIP-1. Once activated by phosphorylation and recruited to the plasma membrane SHIP hydrolyzes the 5' phosphate of phosphatidylinositol (3,4,5)-triphosphate (PIP_3), and of phosphatidylinositol (4,5)-triphosphate (PIP_2) thus preventing the activation of AKT and PLCγ, □□□□□□□□□□□□□□ (37). An intriguing unresolved question is whether and how SLAM receptors can act as inhibitory receptors when SAP is expressed in NK cells. It has been proposed that inhibitory versus activating function of 2B4 in NK cells is regulated by 2B4 receptor expression levels, the availability of intracellular SAP and the density of the CD48 ligand (38). The function of other SLAM family receptors is regulated in similar ways ref.

Another prominent example of a receptor, which is using ITSM for cellular inhibition, is PD1. This receptor is known for its role in limiting T cell mediated damage in situations of chronic stimulation and its presence is a hallmark feature of exhausted T cells. PD-1 is not expressed on normal NK cells but is induced by chronic stimulation such as the presence of multiple myeloma (39, 40) or Hepatitis C Virus infection (40). The cytoplasmic tail of PD-1 contains an ITIM and a switch motif, whereby the ITSM in required for PD-1-dependent inhibition (41). Receptor engagement recruits SHP-1 and SHP-2, but only the latter confers the inhibitory effect by down-regulating PI3K/Akt activity in T cells (42). Thus, ITSM-mediated inhibition may be mediated via more than one pathway and remains to be seen whether distinct types of ITSM-

mediated inhibition are operative in NK cells.

ITIM and switch motif independent NK cell inhibition

There is also emerging evidence for inhibitory signaling pathways that operate independent of ITIM and ITSM motifs. A recent study reported a novel Cbl-b/TAM (TYRO3, AXL and MER) receptor-dependent inhibitory pathway for NK cell activation (43). Engagement of TAM receptor tyrosine kinases with their endogenous ligand Gas6 suppressed proliferation and IFN-γ production by NK cells activated in NKG2D-dependent fashion. Gas6 induced the recruitment of Cbl-b to TAM receptors and their ubiquitinylation, which suppressed NK cell activation by NKG2D and other receptors (**Fig. 1D**). Importantly, *Cbl-b*$^{-/-}$ NK cells were resistant to the GAS6-mediated negative regulation of NK cells, demonstrating that Cbl-b acts downstream of TAM receptors. A possible developmental basis for these defects was excluded by the use of a small-molecule TAM kinase inhibitor, which readily abolished the inhibitory effect of GAS6, and enhanced NK cell cytotoxicity towards cancer cells (43). How the inhibitory Cbl-b/TAM pathway counteracts the function of NK cell activation receptors remains to be determined.

3.) Regulation of NK cell activation at the level of receptor-ligand interaction

Competition between inhibitory ligand expressed in *trans* and *cis*

Inhibitory receptors strongly counteract effector responses when interacting with ligand present on opposing cell membranes (*trans* interaction). However, some inhibitory receptors can also interact with ligand present on the NK cell's membrane (*cis* interaction). This was first described for MHC class I-specific Ly49 receptors in the mouse. A considerable fraction of inhibitory Ly49 receptors is constitutively associated

with MHC ligand in *cis*. These cis complexes appear relatively stable and are not thought to contribute to inhibitory signaling. Thus, cis binding effectively reduces the availability of inhibitory receptors to bind ligand in *trans*. This increases the sensitivity of NK cells to respond to changes of inhibitory or activating ligands on target cells (**Fig. 2A**). Similar cis binding has been reported for the human LILRB1 receptor (44).

Competition between activating and inhibitory receptors for ligand in *trans*

Inhibition can also ensue based on a competition between activating and inhibitory receptors for the same ligand expressed on another cell. The key example for this type of regulation is the competition of co-stimulatory CD28 and co-inhibitory CTLA-4 expressed by T cells for the binding to CD80 and CD86 on antigen presenting cells (APC). Inhibition is based on the upregulation of CTLA-4 upon (chronic) T cell activation and on the fact that its affinity for CD80 and CD86 exceeds that of the co-stimulatory CD28. CD28 and CTLA-4 are also expressed by NK cells, at least in the mouse (45). NK cells up regulate CD28 and CTLA-4 upon culture in IL-2. Activation by plate-bound CD80 induced NK cell IFNγ production, which was largely mediated by CD28 based on the analysis of CD28 deficient NK cells. Conversely, IFNγ production was significantly increased when NK cells lacked CTLA-4 NK cells, indicating that these receptors have opposing roles also in NK cells (45). Thus, competitive mechanisms similar to the ones operating in T cells, may reduce NK cell activation. However, it is not entirely clear to what extent competition and CTLA-4 signaling contribute to the inhibitory effect.

Moreover, NK cells express the activating receptor DNAM-1 (CD226) and the inhibitory receptors T cell Immunoglobulin and ITIM domain (TIGIT) (46) and TACTILE (CD96) (47), which are all specific for CD155 expressed on endothelial cells

and on a variety of immune cell types. Similar to CD28/CTLA-4, the inhibitory receptors (CD96 and TIGIT) have a higher affinity for CD155 as compared to the activating DNAM-1 (46) (**Fig. 2B**). In mice, the absence of CD96 leads to increased NK cell-mediated LPS-induced inflammation and improved tumor rejection, but this effect was only evident when DNAM-1 was expressed. Thus the inhibitory CD96 acts on DNAM-1-induced activation (47). Another intriguing aspect of this system is that CD96 predominantly impacts cytokine production, while TIGIT is mainly responsible for inhibiting killing. If effector functions are indeed impacted selectively rather than globally, it will be important to address at what level these receptors interfere with activation signaling. In this context it may be of importance that DNAM-1-mediated activation signaling depends on its physical association with LFA-1 in the plane NK cell membrane (48).

4.) Non-conventional roles of inhibitory NK cell receptors

The response of individual activation receptors to stimulation can vary significantly between individual NK cells from the same donor. Activation receptors on NK cells, which can recognize MHC class I, respond efficiently to stimulation, while NK cells, which cannot recognize MHC class I respond poorly (10, 49, 50). The improved responsiveness of NK cell activation receptors to stimulation, which depends on inhibitory receptor and ITIM-dependent signals, is referred to as licensing (10). The molecular mechanism for "licensing" is still debated. One possible mechanism, referred to as "disarming", is that activation receptors become by default responsive to stimulation at some point during NK cell development. ITIM-dependent inhibitory signaling would neutralize activation signaling and this would keep the activation pathway competent to respond. If NK cells cannot bind MHC class I, persistent

stimulation would eventually disarm all activation pathways (51). An alternative scenario, referred to as arming, is that NK cell activation receptors are by default poorly responsive to stimulation. The engagement of MHC class I receptors would be needed to render activation receptors responsive (arming) (51). Here, inhibitory receptors and consequently ITIM-dependent signals would instruct NK cell function. The finding that CD94-NKG2A engagement can induce the activity of Abl and phosphorylate Crk (35) is consistent in principle with such a scenario. The improved functionality of activation receptors is essential for efficient NK cell responses to targets that lack MHC class I expression.

Perspectives

The mode of action of inhibitory receptors is still incompletely understood. This applies both to the understanding of intracellular signaling as well extracellular mechanisms in which receptors interfere with cellular activation already at the level of receptor-ligand interactions. In addition, there is some evidence that receptors that were initially identified based on their inhibitory function may perform additional roles. Examples include NK cell licensing, a possible co-stimulatory role of KIR in CD4 T cells (52) and evidence that inhibitory receptors improve NK cell survival (53, 54). The central role of inhibitory receptors is likely to ensure self-tolerance i.e. to prevent NK cell responses to cells that normally express specific markers of "self", such as MHC class I molecules. Inhibitory receptors thus allow "missing-self" responses to aberrant self-cells that have lost the expression of inhibitory ligand. In addition, recent data raise the possibility that inhibitory receptors play a role for the detection of "non-self" cells. NK cells can kill *Cryptococcus* and *Candida*, two fungi, based on NKp30-dependent activation (1). It seems reasonable to assume that NKp30-mediated NK cell activation

can proceed since the fungal cells lack MHC class I molecules and possibly other inhibitory ligands characteristic of human cells. Since, NK cells exert protective roles in other fungal infections, absence of receptor-mediated inhibition may be more important to allow NK cell-mediated recognition of pathogens.

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Figure Legends

Fig. 1. Receptor-mediated inhibitory signaling

A Phosphorylated ITIMs mediate inhibitory signaling by SHP-1 recruitment. A key SHP-1 substrate Vav-1, whose phosphorylation by activation receptors signaling pathways is essential for tight adhesion and the execution of effector functions.

B Inhibitory receptors have been shown to activate c-Abl and lead to the phosphorylation and thus inactivation of Crk. Inhibition is explained by a requirement for NK cell activation via CD16

C In the absence of the small cytosolic adaptor SAP, the ITSM present in 2B4 can initiate inhibitory signaling by recruiting (directly or indirectly) SHIP-1. SHIP hydrolyzes the 5' phosphate from phosphatidylinositol (3,4,5)-triphosphate (PIP_3), thus counteracting the action PI3K and preventing the activation of AKT and PLCγ.

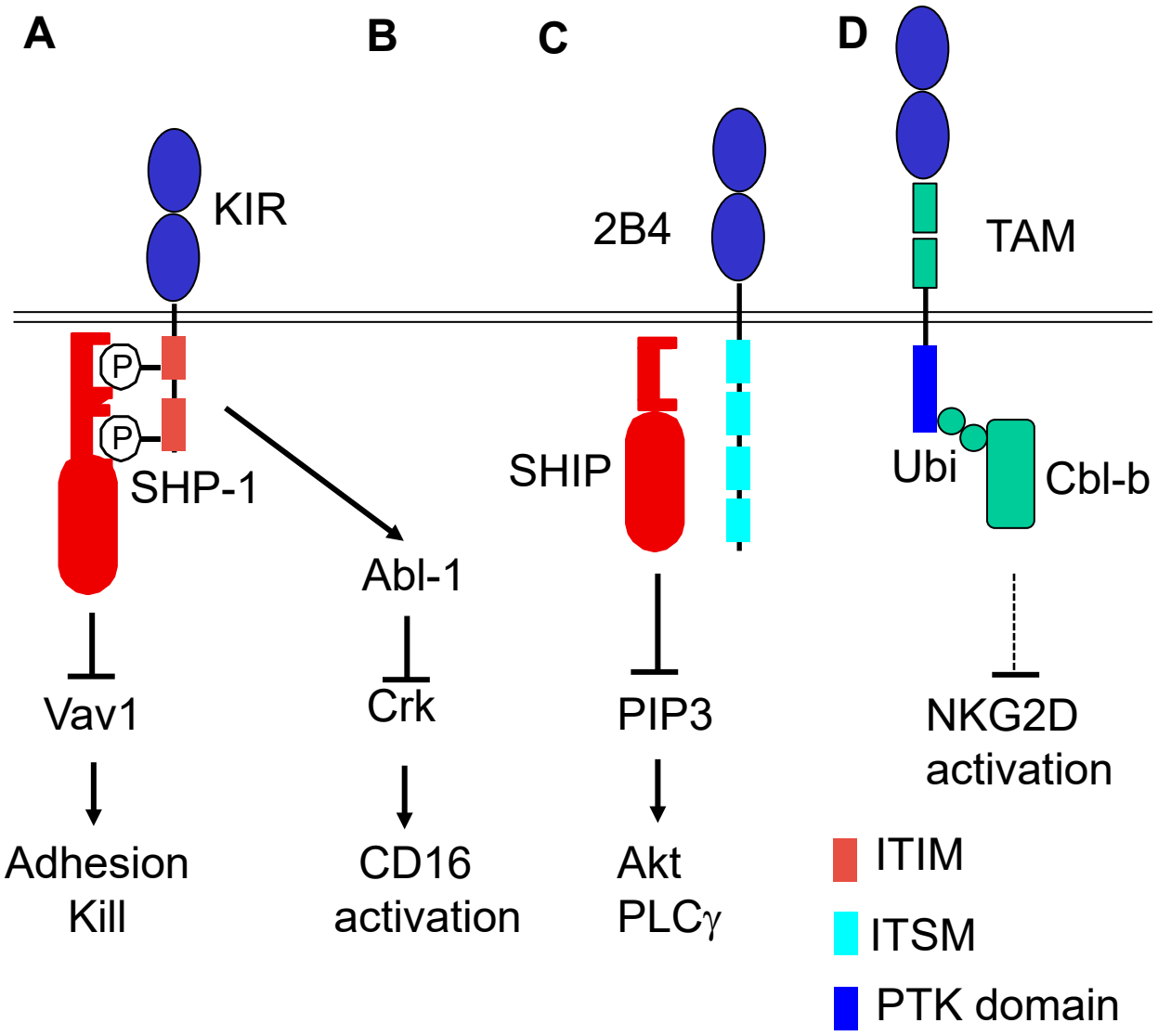
D The engagement of TAM (TYRO3, AXL and MER) receptors induces the recruitment of Cbl-b and the ubiquitinylation (ubi) of the protein tyrosine kinase (PTK) domain of TAM. This suppresses NK cell activation by NKG2D receptors by unknown mechanism.

Fig. 2. Regulation of NK cell activation at the level of receptor-ligand interaction

A A large fraction of inhibitory Ly49 receptors (>70%) is constitutively associated with MHC class I in *cis*. These *cis* complexes do not appear to contribute to inhibitory signaling. Thus, *cis* binding reduces the availability of inhibitory receptors to bind MHC class I in *trans,* thereby lowering the threshold for NK cell activation. The absence of *cis* binding increases the threshold for NK cell activation.

B NK cells can be co-activated by the interaction of the DNAM receptor with CD155 ligand. This is counteracted by a competition between activating DNAM-1 and

inhibitory CD96 or TIGIT for CD155 binding. The activating receptor is disfavored based on a lower affinity for CD155, as indicated by a fainter arrow as compared to the higher affinity CD96/TIGIT binding of CD155.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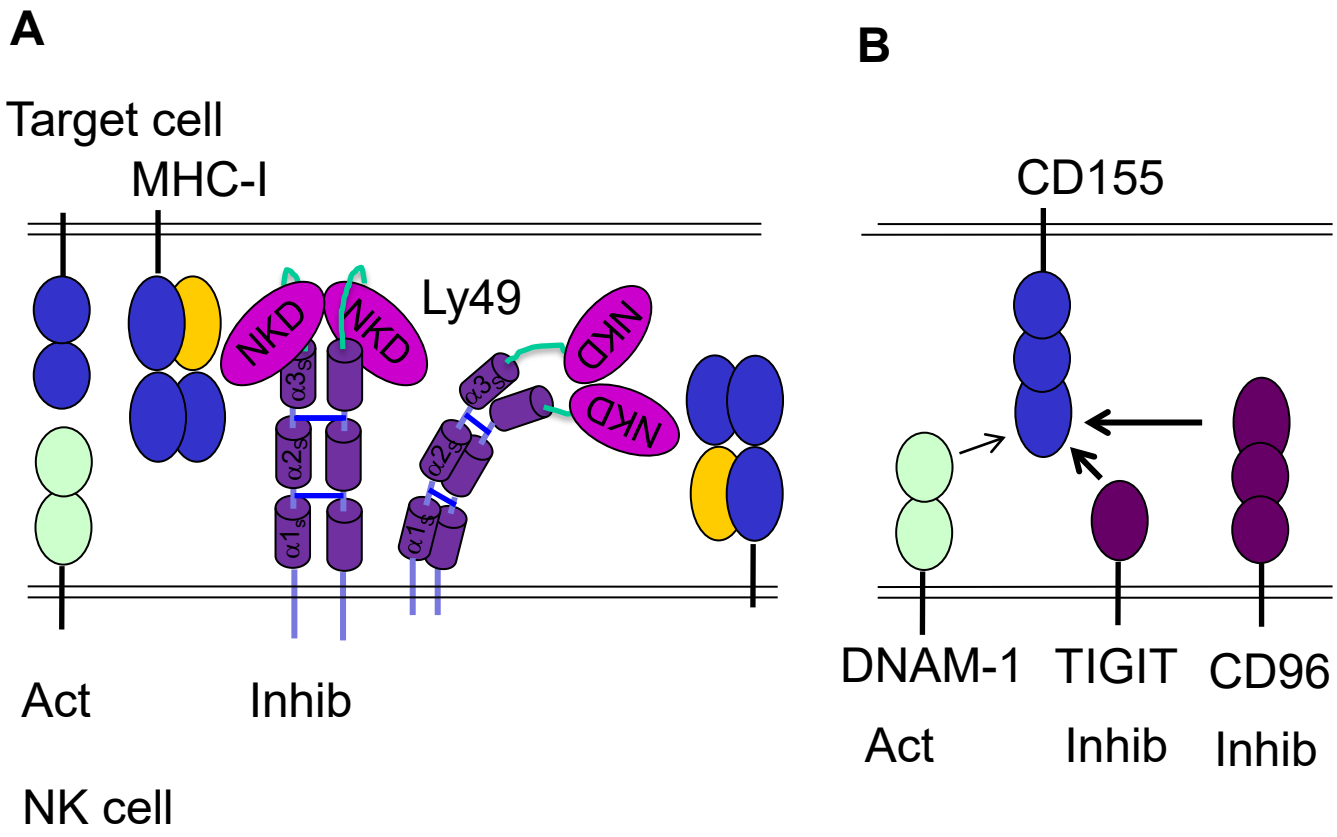
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