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Natural killer cell cytotoxicity and its regulation by inhibitory receptors

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Summary

Natural killer (NK) cells express an array of germ-line encoded receptors that are capable of triggering cytotoxicity. NK cells tend to express many members of a given family of signalling molecules. The presence of many activating receptors and many members of a given family of signalling molecules can enable NK cells to detect different kinds of target cells, and to mount different kinds of responses. This contributes also to the robustness of NK cells responses; cytotoxic functions of NK cells often remain unaffected in the absence of selected signalling molecules. NK cells express many MHC-I-specific inhibitory receptors. Signals from MHC-I-specific inhibitory receptors tightly control NK cell cytotoxicity and, paradoxically, maintain NK cells in a state of proper responsiveness. This review provides a brief overview of the events that underlie NK cell activation, and how signals from inhibitory receptors intercept NK cell activation to prevent inappropriate triggering of cytotoxicity.

Keywords: activating receptor; cytotoxic synapse; immunoreceptor tyrosine-based inhibitory motif (ITIM); inhibitory receptor; inhibitory synapse; killer-cell Ig-like receptor (KIR); natural killer cell.

Introduction

Natural killer (NK) cells are lymphocytes with features of both innate and adaptive immunity. They contribute to immune defense by killing unhealthy cells, secreting soluble factors, and regulating the responses of antigen-presenting cells and the adaptive T cells. NK cells are the predominant lymphocyte population in the uterus, and play important roles in reproduction.[1,2] Unlike adaptive lymphocytes, wherein receptor diversity is generated by DNA rearrangements, NK cells express an array of germ-line encoded receptors capable of triggering activation.[3–8] Despite this difference, NK cells do display the features of adaptive immunity.[9] NK cells can mount a response against infections, including viral, and register a memory for that response. NK cells can distinguish healthy cells from diseased cells. Understanding how specificity is achieved in the recognition of target cells, and how healthy cells are spared from NK cell attack, are among the major goals of NK cell studies. With a focus on human NK cells, this review examines briefly the molecular events of NK cell cytotoxicity, and how NK cell cytotoxicity is controlled to distinguish healthy cells from diseased ones.

NK cell cytotoxicity

Receptors for NK cell cytotoxicity

Natural killer cells express many receptors that can activate their cytotoxic and secretory functions. These receptors recognize ligands on the surface of infected, transformed or stressed cells. The Fc receptor Fcγ RIIIa (CD16) on NK cells recognizes the Fc portion of antibodies, for example, bound to unhealthy cells, and triggers NK cell activation through a process termed as antibody-dependent cell-mediated cytotoxicity.

The natural cytotoxicity receptors (NCR) (NKp30, NKp44 and NKp46) are among the earliest identified NK cell-activating receptors.[10] They are type I transmembrane receptors, which belong to the immunoglobulin superfamily. They are potent inducers of NK cell cytotoxicity, and are important for NK cell-mediated tumour immunosurveillance. They are expressed largely on NK cells. While NKp46 is specific to NK cells, the other two are expressed also on T cell subsets.[11] While NKp46 and NKp30 are expressed on both resting and activated NK cells, expression of NKp44 is induced upon NK cell activation.[12–15] Identification of the cellular ligands of NCRs

has been challenging, except the identification of B7-H6 as an NKp30′s cellular ligand expressed on the surface of tumour cells.[16,17] A number of cellular and viral ligands of these NCRs have been reported, which could trigger or block the receptor.[10,18,19] For example, a nuclear protein BAT3, a ligand of NKp30, could be released from tumour cells in exosomes or as soluble proteins to activate or inhibit NKp30, respectively. The protein CMV pp65 has been described as a viral ligand of NKp30, which binds and inhibits NKp30.

Natural-killer group 2, member D (NKG2D) is a homodimeric C-type lectin-like receptor, which is expressed on the surface of NK cells and cytotoxic T cells.[20] It is a type II transmembrane receptor, belonging to the CD94/NKG2 family.[21,22] NKG2D ligands are many structural homologues of class I MHC, including human ULBPs and MICA/B, and mouse Mult1, H60 and Rae-1.[22,23] The NKG2D ligands are upregulated in infected, stressed and tumour cells, indicating important roles of NKG2D in immune defense against abnormal cells. Tumour cells have been seen to shed NKG2D ligands, which has important implications in tumour immunosurveillance.[24,25]

Natural killer cells express other C-type lectin-like activating receptor NKp80, which is a dimeric type II transmembrane receptor. NKp80 is expressed on all NK cells in the peripheral blood. It recognizes a C-type lectin-like ligand, activation-induced C-type lectin (AICL).[26,27] AICL is upregulated on activated monocytes and NK cells, and NKp80–AICL interaction promotes NK cell-mediated control of monocytes and autologous NK cells.[27]

Natural killer cells also express the members of the signalling lymphocytic activation molecule (SLAM) family of receptors. The SLAM receptor family is a group of type I transmembrane receptors, and has six members.[7,18] NK cells express every member except SLAM (CD150, *SLAMF1*). The other five members are: 2B4 (CD244, *SLAMF4*), Ly-9 (CD229, *SLAMF3*); natural killer, T- and B-cell antigen (NTB-A) or Ly108 (in mouse) (*SLAMF6*); CD84 (*SLAMF5*); and CD2-like receptor-activating cytotoxic cells (CRACC) (CD319, *SLAMF7*). They are expressed on other immune cells too, but not on non-immune cells. The members of SLAM receptor family promote cell–cell interactions through homophilic binding, i.e. they bind themselves in *trans*. The only exception is 2B4, which recognizes another Ig-like molecule CD48 expressed on nearly every haematopoietic cell.[28,29]

DNAX accessory molecule-1 (DNAM-1) is an Ig-like domain-containing receptor.[18,30,31] It is expressed on both mouse and human NK cells. DNAM-1 is also expressed on other immune cells, including CD8+ T cells and myeloid cells. DNAM-1 recognizes CD155 (poliovirus receptor) and CD122 (nectin adhesion molecule),[32] both of which are upregulated on cancerous and virus-infected cells. In human, DNAM-1 associates, physically and functionally, with the β2-integrin LFA-1.[33]

In addition to the above-mentioned receptors, NK cells express many other activating receptors, including CD2, CD44, fractalkine receptor, CD27, CD160, CD137, activating Ly49 receptors (in mouse) and activating killer-cell Ig-like receptors (in human).

Tyr-based motifs for NK cell activation

Most activating receptors of NK cells signal through their cytosolic Tyr-based motifs, and signalling by them initiates with phosphorylation of the key Tyr residues in the motif.[18,34] An assortment of Tyr-based motifs has been described for NK cell activation. The Fc receptor Fc γ RIIIa (CD16) signals upon association with homo- or hetero-dimer of the immunoreceptor Tyr-based activation motif (ITAM)-bearing FcR γ and/or CD3 ζ chains. Similarly, the NCRs NKp30 and NKp46 associate with FcR γ and/or CD3 ζ chains.[10] However, the NCR NKp44 associates with the adaptor protein DAP12,[35] which is homodimeric, with each DAP12 molecule containing a single ITAM.[36,37] DAP12 is the ITAM-bearing partner of the activating killer-cell Ig-like receptors (KIRs) and CD94-NKG2 receptors.[4,38] NKG2D associates with the adaptor molecule DAP10, which bears the activating Tyr-based motif YxxM, which is distinct from the ITAMs.[39] The SLAM family of receptors, such as the best-studied member 2B4, does not require association with a partner chain for the Tyr-based activation motifs. They rather possess a Tyr-based motif S/TxYXXL/I, referred to as immunoreceptor Tyr-based switch motifs (ITSM),[40] in their cytosolic tails, which could signal for NK cell activation.[29] NKp80 also does not require a partner chain for activation motifs, and possesses a motif corresponding to half of an ITAM.[41] Signalling through DNAM-1 requires phosphorylation of a conserved Tyr (Y319 in mouse and Y322 in human) and a conserved Asn (N321 in mouse and N324 in human) that are present in its cytosolic tail.[30]

Signalling pathways of NK cell-activating receptors

Because ITAMs are utilized by many immunoreceptors, including the T cell receptor (TCR), ITAM-based signalling is among the best-understood pathways.[42] ITAMs are phosphorylated by the Src family kinases. The phosphorylated ITAMs, through SH2 domain-based interactions, recruit the Tyr kinases ZAP-70 and Syk, which, in turn, could phosphorylate transmembrane adaptor proteins, leading to recruitment of several signalling molecules, including the phosphoinositide 3-kinase (PI3K), phospholipase C (PLC)-γ1 and -γ2, and the guanine nucleotide exchange factors Vav-1, 2, 3. The importance

of different isoforms of PLC-γ and Vav could be different for different ITAM-bearing receptors.[43–46]

Signalling by NKG2D occurs through DAP10, and does not require the Tyr kinases ZAP-70 or Syk.[47–49] The YxxM motif of DAP10 could be phosphorylated by Src family kinases, and could bind to either PI3K or the cytosolic adaptor protein Grb2. The p85 subunit of PI3K can bind to the cytosolic adaptor proteins CrkL and Grb2, and to the guanine nucleotide exchange factor Vav-1, leading to the activation of GTPases to promote NK cell-target cell adhesion and synapse formation.[47,50–53]

2B4, a member of the SLAM family of receptors, is among the best-understood NK cell-activating receptors.[7] SLAM receptor's ITSMs can be phosphorylated by Src family kinases. Phosphorylated ITSM recruits the SAP family of small cytosolic adaptor proteins, SLAM-associated protein (SAP), Ewing's sarcoma-associated transcript-2 (EAT-2) or EAT-2-related transducer (ERT).[54–57] It appears that both SAP and EAT-2 contribute to NK cell activation by 2B4. However, NK cell activation by CRACC is SAP-independent and requires EAT-2.[58] The SAP family adaptors are composed largely of an SH2 domain. SAP could contribute to SLAM receptor-mediated NK cell activation by preventing the recruitment of SH2-domain containing inositol 5′ phosphatase-1 (SHIP-1).[59] The SAP family adaptors could prevent the recruitment of SHIP, the protein Tyr phosphatases SHP-1/2 and the protein Tyr kinase Csk.[60,61] Further, SAP family adaptors could also recruit specific molecules to elicit signalling by SLAM receptors. For example, SAP promotes activation of mouse NK cells by Fyn-induced phosphorylation of Vav-1.[59]

The conserved Tyr- and Asn-based motif of DNAM-1 could be phosphorylated by Src family kinases, which, upon phosphorylation, could recruit Grb2.[30] In this respect, DNAM-1 is similar to the YxxM motif of DAP10. The Met residue at the position +3 (relative to the key Tyr residue) of the YxxM motif is essential for the recruitment of the p85 subunit of PI3K. DNAM-1 lacks this +3 Met residue, but yet recruits p85, seemingly through an indirect mode. The signalling outcomes of such differences in recruiting the same set of molecules by different activation receptors are unclear.

CD2 possesses Pro-rich sequences in its cytosolic tail, which are seen to interact with the Src family kinase Lck and many adaptors.[62] How exactly signalling is achieved by CD2 is unclear.

NK cell cytotoxic synapse

When an NK cell recognizes a sensitive target cell, it makes a cell–cell junction with the target cell. The synaptic cleft formed at the cell–cell junction is mediated by engagements of many transmembrane receptors with their cognate cell surface ligands. This creates a highly organized intercellular junction, called immunological synapse, for vectoral communication between the cells.[63] Immunological synapses accumulate and integrate signals from the engaged receptors to determine the signalling outcomes.[63,64]

Signalling at NK cell cytotoxic synapses, in response to sensitive target cells, triggers a series of cell biological processes that culminates in cytolytic degranulation towards the target cells and secretion of soluble factors.[65] After initial NK cell-target cell adhesion, the lytic granules of NK cells dock onto the microtubule-organizing centre (MTOC) in a process termed granule convergence. Then, the MTOC, along with the docked lytic granules, is polarized towards the NK-target cell synapses. This process is known as granule polarization, and is associated with dramatic cytoskeletal rearrangements, including actin accumulation at the synapses. A fraction of the polarized lytic granules traverses through the actin meshwork[66,67] at the synapse, and exocytose their lytic contents (degranulation) that leads to target cell killing. It appears that the requirement of signal strength is different for different NK cell responses, such as degranulation versus cytokine secretion.[3]

Natural killer cells, like cytotoxic T cells, require signalling by the β2-integrin LFA-1 (αLβ2, CD11a/CD18), for adhesion to target cells and proper cytotoxicity. LFA-1 recognizes ICAM-1 on target cells. In primary T cells, LFA-1 requires activation by inside-out signals, from TCR or a chemokine receptor, in order to bind ICAM-1.[68,69] Contrarily, in NK cells, LFA-1 molecules exit in intermediate conformations, which can bind ICAM-1 and signal autonomously.[70,71] Therefore, it has been possible to dissect out the signalling properties of LFA-1 in the absence of other signals. It is seen that LFA-1 signalling is sufficient to induce signals for actin reorganization and granule polarization in human NK cells.[72,73]

After the initial contact of NK cells with the target cells, LFA-1 appears to initiate the process of synapse formation. Using human NK cells, it is seen that many of the signalling molecules, including PLC-γ and Syk kinase, that are phosphorylated upon CD16 engagement, are also phosphorylated in response to LFA-1 engagement.[74] Despite this overlap, LFA-1 signalling fails to induce Ca^{2+} mobilization, and CD16 signalling, although induces Ca^{2+} mobilization, cannot lead to granule polarization. A recent study with human NK cells, using proteomics, has identified signals that emerge upon LFA-1 engagement, and has validated candidate molecules for their roles in granule polarization.[75] It was seen that LFA-1-induced granule polarization utilizes a conserved set of signalling events, involving an integrin-linked kinase ILK, Pyk2, Paxillin and RhoGEF7, which are also used to establish cell polarity during migration. Further, using a human NK cell line that has granules pre-converged at the MTOC, it was seen that Pyk2, Leupaxin, Cdc42 or CLIP-

170 is required, and ILK or RhoGEF7 is not required, for granule convergence.[75]

LFA-1 alone fails to induce degranulation. It is possible that LFA-1-mediated adhesion and granule polarization are transient processes, unless stabilized by signals from activating receptors and/or not intercepted by inhibitory signals (see below). Experiments involving activation of primary human NK cells by insect cells expressing ligands of individual or combination of receptors (involving NKG2D, DNAM-1, 2B4, CD2 and LFA-1) have shown that each activating receptor alone can elicit inside-out signal for LFA-1 activation but none, except CD16, could induce degranulation at its own.[76] CD16 alone or a synergistic pair of activating receptors could induce degranulation, but without polarization.[73] Such unpolarized degranulation is not sufficient for efficient target cell lysis. Polarized degranulation towards a target cell could be achieved when LFA-1 is engaged together with CD16 or a synergistic receptor pair. Therefore, a collective engagement of LFA-1 and a synergistic receptor pair (or CD16 alone) represents the minimal requirement to trigger NK cell cytotoxicity.[76]

Receptor synergy for inducing secretion by NK cells appears more complex.[77] For example, in human NK cells, 2B4, which alone cannot induce degranulation, could induce IFN-γ at its own. However, 2B4 can induce higher IFN-γ secretion in the presence of a synergistic partner.[77] The molecular basis of synergy among NK cell-activating receptors is not well understood.[78,79] The requirement of synergistic receptor pairs could maintain a high threshold for NK cell activation, and thus could serve as a safeguard mechanism to ensure restrained activation. CD16 induces NK cell activation through its interaction with the Fc portion of antibodies, and thus the adaptive arm of the immune system determines specificity here. This could be the reason why CD16 does not require a synergistic partner for inducing degranulation.

Studying synapses formed between NK cells and target cells, it is seen that adhesion molecules, including LFA-1, quickly segregate to the periphery of the synapses,[80–82] where they contribute to adhesion as well as activation signals for F-actin accumulation. Actin polymerization and its synaptic accumulation are necessary for the formation of NK cell activation synapses and NK cell cytotoxicity.[72,80,83] Similar peripheral distribution of LFA-1 is seen at T cell immune synapses.[84] Perforin is seen to accumulate in the central region of the NK cell activation synapses. It appears that perforin accumulation follows F-actin and adhesion molecule accumulation at the synapses.[80] Further, accumulation of adhesion molecules at the synapses requires actin, but not microtubule functions.[65] On the other hand, as expected, perforin accumulation requires microtubule functions.[65]

While LFA-1 accumulates at the periphery of the NK cell activation synapses, different distributions of activating receptors are seen. A recent investigation has studied NK cell activation synapses formed by applying human NK cells onto supported lipid bilayer carrying ligands for LFA-1 and/or NKG2D-2B4 synergistic pair of activating receptors, as well as those formed between NK cells and target cells.[85] It is seen that LFA-1 controls the distribution of the synergistic pair of the activating receptors. In the absence of LFA-1 engagement, 2B4 and NKG2D form separate clusters in a non-concentric manner. However, co-engagement of LFA-1 imposes a distinct distribution, wherein 2B4 accumulates at the centre of the synapses, while NKG2D accumulates at the periphery. This spatial segregation of this synergistic receptor pair of NK cells is reminiscent of that seen for TCR-CD28 and TCR-CD2 pairs at T cell activation synapses.[86,87] Further, LFA-1 could control synaptic accumulation of CD16. Thus, it appears that formation of organized receptor–ligand pairs at NK cell activation synapses is dependent on LFA-1.[85] Further, the initial accumulation of LFA-1 at the periphery of NK cell cytotoxic synapses seems to create a confined central zone for degranulation and membrane retrieval.

Inhibition of NK cell cytotoxicity

Natural killer cells possess a vast array of germ-line encoded receptors that can trigger cytotoxicity. This immediately raises the questions as to how NK cells achieve specificity in recognition of target cells and how healthy cells are spared from NK cell cytotoxicity. As noted above, individual activating receptors fail in triggering NK cell cytotoxicity, and at least a pair of receptors, that provides synergistic signals, is required for triggering NK cell activation.[78,79] This requirement of synergistic pairs of receptors for NK cell activation could be a safeguard mechanism to avoid unrestrained NK cell cytotoxicity. Further, many of the ligands of NK cell activating receptors are expressed specifically on unhealthy cells, which could ensure specific killing of unhealthy cells. However, ligands of NK cell-activating receptors can be expressed constitutively on healthy cells. For example, normal human articular chondrocytes express a ligand for NKp44.[88] The most important regulation of NK cell activation is provided by MHC-I-specific inhibitory receptors.[89] The activation of NK cells is tightly controlled by signals from MHC-I-specific inhibitory receptors. MHC-I-expressing healthy cells, therefore, could avoid NK cell attack by engaging those MHC-I-specific inhibitory receptors on NK cells, and thereby triggering inhibition of cytotoxicity. Signalling by inhibitory receptors also provides NK cells a proper responsiveness via a process termed as licensing; NK cells lacking inhibitory receptors are hyporesponsive.[90] Aged T cells could acquire these inhibitory receptors, which may dampen T cell responses in aged individuals.[91]

MHC-I-specific receptors for inhibition of NK cell activation

Human NK cells express two major classes of inhibitory receptors, the inhibitory members of KIR and the CD94-NKG2A heterodimer. While NKG2A is expressed in both mouse and human, KIR is expressed in human and not in mouse. KIR is a type I transmembrane receptor possessing extracellular Ig-like domains. NKG2A is a type II transmembrane receptor, belonging to the CD94-NKG2 C-type lectin family. While KIR recognizes HLA-C expression, NKG2A recognizes the expression of the non-classical HLA-E on other cells. Both these classes of inhibitory receptors signal through immunoreceptor Tyr-based inhibitory motif (ITIM), which has a consensus amino acid sequence of V/IxYxxL/V.[89,92–94] Unlike the mouse MHC-I-specific Ly49 inhibitory receptors,[95] which possess only one ITIM, KIR as well as NKG2A possess two ITIMs that are separated by ~26 amino acid residues. In the case of the mouse Ly49 receptor, it is likely that receptor homo-dimerization provides two ITIMs.

Immunoreceptor Tyr-based inhibitory motif-dependent inhibition was first seen in mouse with the receptor FcγRIIb, which inhibits B cell receptor signalling by recruiting the inositol phosphatase SHIP.[96] However, most of the ITIM-bearing receptors recruit the Tyr phosphatase SHP-1 or SHP-2, not SHIP. The ITIM-dependent inhibition of NK cell activation prefers recruitment of SHP-1 to the ITIMs.[94,97,98] SHP-1 is composed of two tandem SH2 domains followed by a catalytic domain and a C-terminal tail. SHP-1 is maintained in an auto-inhibited conformation in the cytosol, wherein its N-terminal SH2 domain docks onto its catalytic domain.[99] Binding of SHP's tandem SH2 domains with ITIMs can relieve the auto-inhibited conformation to activate its catalytic activity.[100]

Signalling pathways of MHC-I-specific inhibitory receptors of NK cells

It is not known which kinase phosphorylates ITIMs; the Src family kinases Lck and Fyn are the candidates.[94,101,102] The two phospho-ITIMs (pITIM) that are separated by ~26 amino acid residues provide specific binding sites for the two tandem SH2 domains of SHP-1. These interactions could break the interaction of SHP-1′s N-terminal SH2 domain with its catalytic domain, and thus could relieve the auto-inhibited conformation of SHP-1. According to the initial view, activated SHP-1 may dephosphorylate multiple signalling molecules to block activation.[89] However, Vav-1 is the only major substrate identified directly from NK cell inhibition (Fig. 1).[103] Vav-1 is essential for TCR-mediated signals for Ca^{2+} mobilization, actin remodelling and synapse formation.[104] Therefore, SHP-1-mediated dephosphorylation of activating Tyr residues of Vav-1 could be sufficient, and dephosphorylation of multiple activating molecules may not be necessary for inhibition.

Inhibitory receptors are seen to co-cluster with activating receptors and invoke inhibition locally[81,105–107] without impairing the ability of NK cells to respond to other

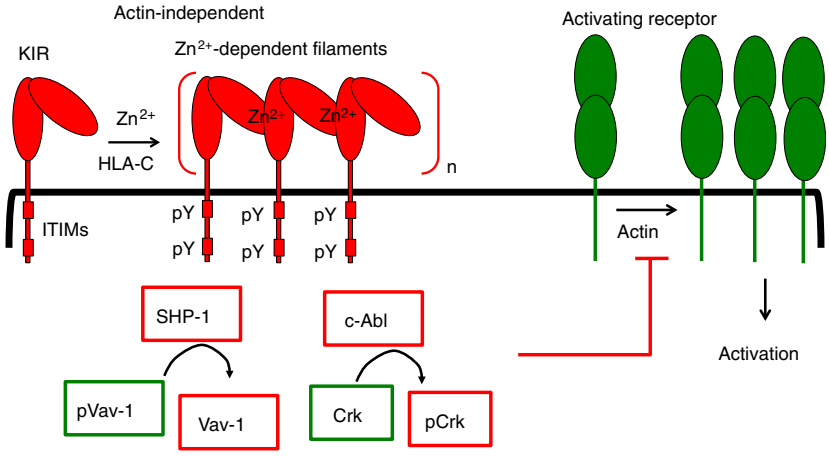

Figure 1. Interception of natural killer (NK) cell activation by killer-cell Ig-like receptor (KIR). At the inhibitory synapses formed between KIR+ NK cells and HLA-C+ target cells, KIR clusters rapidly, in actin-independent manner. The zinc-dependent polymerization of KIR into filaments could contribute to the rapid and actin-independent KIR clustering at these synapses. The Src family kinase Lck and Fyn are candidate kinases for immunoreceptor Tyr-based inhibitory motif (ITIM) phosphorylation. The protein Tyr phosphates SHP-1, recruited and activated by its interaction with phospho-ITIMs (pITIMs), dephosphorylates the guanine nucleotide exchange factor Vav-1. The c-Abl kinase is recruited to the inhibitory synapses through an unknown mechanism. The c-Abl kinase phosphorylates the small adaptor protein Crk, and dissociates it from a signalling complex (not shown here) formed during activation. Vav-1 dephosphorylation and Crk phosphorylation contribute to blockage of actin-dependent signals for NK cell activation, and thus could contribute to inhibition of proximal actin-dependent steps, such as LFA-1 activation (not shown here) and clustering of activating receptors.

stimuli. This led to the term co-inhibition, and the inhibitory receptors were considered as co-receptors. However, it is now clear that the NK cell inhibitory receptors can signal independently.[108] During inhibition by ITIM-bearing receptor of human NK cells, a small adaptor protein Crk becomes phosphorylated and associates with the protein Tyr kinase c-Abl.[108,109] During activation of human NK cells, Crk is seen to be associated with signalling complexes involving c-Cbl, C3G and $p130^{CAS}$. As a result of its phosphorylation during inhibition, Crk dissociates from these complexes. Experiments involving human NK cells on ligand-attached lipid bilayer have shown that HLA-E alone on the lipid bilayer can induce Crk phosphorylation, indicating that ITIM-bearing inhibitory receptors can signal independently.[108] The signalling complexes involving Crk, c-Cbl, C3G and $p130^{CAS}$ are seen to control cytoskeletal-remodelling.[110–112] Crk is seen to be required for different steps of NK cell cytotoxicity.[113] Crk is required for CD16 signalling in human NK cells on Fc-containing lipid bilayer; silencing of Crk impairs movement of Fc clusters and reduces CD16-induced Vav-1 phosphorylation. Co-engagement of HLA-E with CD16 induces Crk phosphorylation and prevents actin-dependent signals in human NK cells.[108] A membrane-target Crk, which lacks the Tyr residues that are substrates of c-Abl kinase, partially relieves KIR-mediated inhibition of NK cells.[109] These observations suggest that Crk phosphorylation contributes to NK cell inhibition (Fig. 1).

In summary, NK cell inhibitory receptors can signal independently, and they signal through two pathways (Fig. 1). One pathway involves Vav-1 dephosphorylation by SHP-1. The other pathway does not involve dephosphorylation of activating molecules. Rather, it involves c-Abl-mediated phosphorylation of Crk and its dissociation from signalling complexes formed during activation. Both Vav-1 dephosphorylation and Crk phosphorylation can contribute to inhibition of actin-dependent signals of activation.

NK cell Inhibitory synapses

Upon binding to MHC-I ligands on another cell, inhibitory receptors of an NK cell cluster at the inhibitory synapse formed between the two cell types.[114] The amount of KIR accumulated at the synapse is seen to be proportional to the number of KIR molecules engaged with HLA ligands.[115] Accordingly, inhibition of killing increases with an increase in the expression level of HLA ligands on the target cells.[115] NK cell inhibitory receptors possess unusual features. Clustering of KIR and NKG2A is independent of actin-related processes and ATP.[114,116] KIR accumulation at inhibitory synapses is independent of ITIM signalling, ICAM-1-mediated adhesion, or any other vertebrate molecule on the target cell.[116,117] Interestingly, KIR clustering requires the metal ion Zn^{2+} (see below).

Upon binding with HLA-C on target cells, KIR is seen to cluster quickly at the centre of the inhibitory synapses along with some SHP-1 molecules.[107,114] The centre of the inhibitory synapses also accumulates some GM1 sphingolipid, suggesting accumulation of activating receptors, which do not spatially overlap with KIR clusters.[107] At this early time, LFA-1 localizes at the periphery of the synapses, encircling the centrally accumulated KIR. As the inhibitory synapses further develop, this specific pattern of synaptic organization is disrupted and GM1 or LFA-1 accumulation is blocked.[107]

It is seen that the organization of inhibitory synapses depends on the amount of KIR engagement.[115] When a lower number of KIR molecules on NK cells is engaged with HLA-C on target cells, numerous small KIR microclusters are seen at the inhibitory synapses. However, at a higher level of KIR engagement, mixed patterns of KIR clustering are seen, where the majority of synapses form a homogenous large KIR cluster. This phenomenon is independent of actin-dependent processes. The relative spatial localization of KIR and LFA-1 at inhibitory synapses is also seen to depend on the level of KIR engagement with HLA-C.[115] The extent of segregation between the KIR and LFA-1 clusters is seen to be proportional to the number of KIR molecules on NK cells that are engaged with HLA-C on target cells. The extent of segregation of LFA-1 and KIR clusters correlates positively with the extent of inhibition of NK cell-mediated killing of target cells.[115] Therefore, it appears that the number of engaged inhibitory receptors controls the synaptic organization as well as inhibitory function of the receptor.

Signalling-competent NK cell inhibitory synapses can be reconstituted on a planar lipid bilayer carrying only HLA-E, suggesting that NK cell inhibitory receptors can signal independently.[108] On lipid bilayers carrying HLA-E alone, HLA-E initially forms a peripheral ring, which rapidly moves towards the centre of the synapse. On lipid bilayers carrying Fc only, Fc initially forms numerous microclusters that move centripetally to accumulate at the centre. On lipid bilayers carrying both HLA-E and Fc, they are seen to form a few microclusters and to co-localize to the centre of the synapse. Fewer Fc microclusters are formed in the presence of HLA-E, but the central accumulation of Fc microclusters is not impaired in the presence of HLA-E. Therefore, NKG2A engagement prevents formation of CD16 microclusters.[108] At the T cell activation synapses too, early TCR microclusters are formed that later converge into a central cluster, and these TCR microclusters are the sites of active signalling.[118] Conceivably, CD16 microclusters contribute to signalling, and thus NKG2A-mediated reduction in the number of Fc microclusters would prevent CD16 signalling.

It appears from these studies that activation and inhibitory receptors initially accumulate at the periphery and later converge at the centre of inhibitory synapses, LFA-1 appears to encircle the centrally accumulated activation and inhibitory receptors, and inhibitory receptors prevent further accumulation of LFA-1 and activating receptors.

Lck and Fyn are candidate kinases for ITIM phosphorylation. How the phosphorylation of KIR's ITIMs is initiated and controlled is not understood. A H36A mutation in KIR results in constitutive phosphorylation and self-association of the receptor.[119] It appears that KIR self-association makes its cytosolic ITIMs inaccessible to phosphatases, which favours ITIM phosphorylation. It is proposed that ITIM phosphorylation is controlled by KIR self-association, and that His36 acts as a gatekeeper to avoid uncontrolled KIR signalling.[119] In the kinetic partitioning model[120] of TCR phosphorylation, ITAM phosphorylation is controlled by a size-based exclusion of the transmembrane phosphatase CD45 from the central synaptic zones. The receptor self-association model[119] is similar, wherein phosphorylation is achieved by protecting the phosphorylated Tyr residues from phosphatases. However, in the receptor self-association model of KIR phosphorylation, the receptor itself converts into a state that protects ITIM's Tyr residues from local phosphatases.

Killer-cell Ig-like receptor phosphorylation is localized to the synaptic region and does not spread to the extrasynaptic regions of the plasma membrane.[105] Thus, NK cell inhibition is local and not cell-wide. Further, phosphorylated KIR molecules are not distributed uniformly; rather, they are localized in discrete microclusters within the larger KIR cluster at the inhibitory synapses.[105] In a recent study with super-resolution microscopy, it is seen that KIR organizes into nanoclusters, and that SHP-1 preferentially associates with larger KIR nanoclusters.[121] The implication of such distribution of phosphorylated KIR at inhibitory synapses is not understood.

Interception of NK cell activation pathways by signals from inhibitory receptors

As noted above, signals for granule polarization and degranulation can be uncoupled in NK cells.[73,76,78] LFA-1 alone can induce granule polarization without degranulation, and CD16 alone (or a synergistic activating receptor pair) can induce degranulation without polarization of granules towards the target cells. While inhibition of degranulation appears to have much more stringent requirements, inhibitory receptors efficiently prevent LFA-1-induced granule polarization, indicating that granule polarization is highly sensitive to inhibition.[122]

Inhibitory signals can intersect NK cell activation at multiple points, including adhesion to target cells, inside-out signals for LFA-1 activation, clustering of activating receptors, synaptic accumulation of F-actin, and Ca^{2+} flux.[65] These steps are actin-dependent, which can be blocked by Vav-1 dephosphorylation and/or Crk phosphorylation during inhibition (Fig. 1). The movement of lytic granules along microtubules and their accumulation at the MTOC depend on dynein, not on actin or microtubule reorganization. Interestingly, this granule convergence, which is independent of actin or microtubule reorganization, is not sensitive to signals from these inhibitory receptors.[123]

Natural killer cell inhibitory receptors have an ability to accumulate rapidly at inhibitory synapses.[124] This rapid accumulation at inhibitory synapses, which is independent of actin, may confer to these inhibitory receptors ability of blocking proximal activation signals, such as actin-dependent adhesion, LFA-1 activation and clustering of activating receptors, before the full cascade for activation.[76,124,125] By blocking proximal activation signals, signals from NK cell inhibitory receptors tend to dominate over those from activating receptors. Signalling by chemokine receptor for fractalkine (CX3CL1) is the only example, apart from activation by soluble molecules, which is not sensitive to the signals from inhibitory receptors.[126] The signalling properties of the fractalkine receptor, which confer to the receptor this unusual feature, are not understood.

Therefore, control of NK cell cytotoxicity is not a simple balance between signals from activation and inhibitory receptors. Rather, the physiological outcome, of the co-engagement of NK cell activation and inhibitory receptors, is tilted towards the inhibitory signals. However, it is not clear as to how NK cell inhibitory receptors could achieve a rapid actin-independent clustering and signalling.

A role of Zn^{2+} at NK cell inhibitory synapses

The inhibitory function of KIR has long been known to be dependent on Zn^{2+}.[127] The primary amino acid sequence of KIR is rich in His residues, including the N-terminal HExxH zinc-binding motif, which can bind to Zn^{2+}.[128,129] While the clustering and functioning of KIR at NK cell inhibitory synapses are independent of actin or ATP, they require Zn^{2+}.[114] Zn^{2+} does not contribute to the binding of KIR to HLA-C.[128]

How Zn^{2+} contributes to clustering and signalling of KIR is not understood. Zn^{2+} induces self-association of soluble KIR (purified ectodomain of KIR) into filamentous polymers.[130] The soluble KIR possesses three Trp residues (at positions 29, 188 and 207) that span the entire length of the protein. The Zn^{2+}-induced KIR polymerization is coupled with a change in Trp environment, suggesting a Zn^{2+}-induced conformational change in KIR. Zn^{2+} treatment of KIR expressing NK cells leads to masking of KIR epitopes for antibody as well as HLA-C

binding, which could arise from Zn^{2+}-induced conformational change in the receptor and/or receptor polymerization in NK cells. Similar KIR filaments are isolated from the lysate of Zn^{2+}-treated NK cells. KIR filaments form spontaneously, without addition of exogenous Zn^{2+}, at functional inhibitory synapses formed between KIR expressing NK cells and HLA-C expressing target cells. Zn^{2+} treatment of NK cells expressing Venus-tagged KIR leads to a decrease in the fluorescence anisotropy of Venus, indicating KIR self-association at the surface of intact NK cells. Two independent mutations in KIR's extracellular portion, which are away from the HLA-C binding site and do not impair HLA-C binding, impair Zn^{2+}-induced polymerization and inhibitory function. These results indicate that KIR undergoes Zn^{2+}-dependent polymerization during its signalling, and that Zn^{2+}-dependent polymerization is required for KIR's inhibitory function.[130] However, Zn^{2+} alone is not sufficient, and HLA-C binding is required, to induce ITIM phosphorylation.

Zn^{2+}-induced polymerization of KIR represents a new mode of transmembrane receptor signalling, wherein a transmembrane receptor polymerizes into higher-order assemblies that are much larger than the known dimers and oligomers of transmembrane receptors.[130] Zn^{2+}-dependent KIR polymerization represents a new role for Zn^{2+} in the regulation of receptor signalling at the plasma membrane. KIR is organized into nanoclusters at the plasma membrane of resting NK cells,[121] which could promote rapid Zn^{2+}-dependent polymerization. Thereby, conceivably, Zn^{2+}-induced polymerization could contribute to the rapid, actin-independent KIR clustering and signalling at inhibitory synapses.

Many signalling pathways involve assembly of cytosolic molecules into supramolecular assemblies.[131,132] Zn^{2+}-dependent KIR polymerization advances this paradigm of signalling by supramolecular assemblies by providing the first example of a transmembrane receptor that signals upon polymerization. Protein assembly is often described by a nucleation-dependent polymerization (NDP) mechanism,[133,134] wherein the initial steps (nucleation) are slower than the later ones. In the case of KIR, it is conceivable that the proximity of KIR molecules in the nanoclusters[121] and involvement of Zn^{2+}[130] make the nucleation favourable to achieve a rapid polymerization. Assembly of supramolecular signalling complexes via NDP mechanism may invoke new mechanisms of signal amplification, setting threshold and spatio-temporal regulation in signal transduction.[132] While principles of protein polymerization[134] predict potential implications of assembly of supramolecular signalling complexes in signal transduction, whether and how a transmembrane receptor can control these assembly reactions in the cytosol are unclear. KIR offers a model system to understand this.

Summary

Natural killer cells possess many receptors that can trigger their cytotoxic functions. Many of these receptors signal through ITAM-dependent and many through ITAM-independent pathways. The receptors that signal through ITAM-dependent pathways associate with ITAM-containing chains, such as FcRγ, CD3ζ and DAP12. NKG2D signals through its association with DAP10, which possesses the YxxM motif that is distinct from ITAM. SLAM family of receptors signals through their cytosolic ITSM. Utilization of different kinds of signalling pathways could explain why NK cells tend to possess several members of a given family of signalling molecules. These different kinds of activating receptors and many members of a given family of signalling molecules may enable NK cells to detect different kinds of target cells and to mount different kinds of responses. The β2-integrin LFA-1, which accumulates at the periphery of cytotoxic synapses, appears to play a pivotal role in organizing the signalling complexes and creating a confined central zone for degranulation at the NK cell cytotoxic synapses. A major goal of current NK cell biology studies is to understand the signalling properties of different activating receptors and how signals from multiple receptors are integrated to mount NK cell responses.

Natural killer cell cytotoxicity is tightly controlled by the dominant inhibition exerted by MHC-I-specific inhibitory receptors. The MHC-I-specific inhibitory receptors of human NK cells, KIR and NKG2A, signal through their cytosolic ITIMs. Phosphorylated ITIMs recruit and activate the protein Tyr phosphatase SHP-1, which dephosphorylates the guanine nucleotide exchange factor Vav-1. During inhibition, the small adaptor protein Crk becomes phosphorylated, dissociates from the signalling complexes formed during activation, and associates with the c-Abl kinase. The dephosphorylation of Vav-1 and phosphorylation of Crk contribute to inhibition. Future investigations should focus on how these components of inhibitory signalling pathways operate and coordinate at NK cell inhibitory synapses. Are there more ITIM-dependent pathways for interception of activation signals and/or for licensing of NK cells? KIR signalling requires its Zn^{2+}-dependent polymerization at inhibitory synapses. An important future goal would be to elucidate how Zn^{2+} is delivered at NK cell inhibitory synapses and how Zn^{2+}-dependent polymers contribute to KIR signalling.

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Disclosures

There is no conflict of interest

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