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Up on the tightrope: natural killer cell activation and inhibition

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Abstract

Natural killer (NK) cells circulate through the blood, lymphatics and tissues, on patrol for the presence of transformed or pathogen-infected cells. As almost all NK cell receptors bind to host-encoded ligands, signals are constantly being transmitted into NK cells, whether they interact with normal or abnormal cells. The sophisticated repertoire of activating and inhibitory receptors that has evolved to regulate NK cell activity ensures that NK cells protect hosts against pathogens, yet prevents deleterious NK cell—driven autoimmune responses. Here I highlight recent advances in our understanding of the structural properties and signaling pathways of the inhibitory and activating NK cell receptors, with a particular focus on the ITAM-dependent activating receptors, the NKG2D-DAP10 receptor complexes and the CD244 receptor system.

NK cells, like other members of the innate immune system, were once considered nonspecific in their interactions with tumors or virus-infected cells. A seminal study noted that NK cells can kill certain tumor cells lacking MHC class I, yet spare the same tumors expressing MHC class I (ref. 1). This ability to attack cells ‘missing self’ predicted the existence of inhibitory receptors on NK cells, and it revealed the mechanism whereby NK cells engage in surveillance for transformed or virus-infected cells that have downregulated expression of MHC class I in an effort to avoid recognition by $CD8^+$ cytotoxic T lymphocytes. However, missing from the missing-self hypothesis was any molecular basis for how NK cells are activated when they encounter tumors or virus-infected cells, or how in certain circumstances NK cells do efficiently recognize and kill tumors or virus-infected cells that maintain expression of MHC class I. Even after the discovery of the inhibitory NK cell receptors, activation of NK cells against MHC class I—deficient cells was generally considered nonspecific. Not so anymore. Many activating NK cell receptors and pathways have recently been identified, and the interplay between the inhibitory receptors and activating receptors are being defined. This review highlights the predominant signaling pathways that regulate the responses of NK cells encountering potential target cells.

Inhibitory NK receptors

NK cells express a repertoire of inhibitory receptors that regulate their activation. Some inhibitory NK receptors are specific for MHC class I, whereas others bind non-MHC ligands (Supplementary Table 1 online). Some of these inhibitory NK receptors, such as killer cell immunoglobulin-like receptors (KIRs) and leukocyte immunoglobulin-like receptors (LILRs), are monomeric type I glycoproteins of the immunoglobulin superfamily, whereas others, such

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as the Ly49 and CD94-NKG2A receptors, are type II glycoproteins with a C-type lectin—like scaffold. Although diverse in their extracellular domains, these inhibitory NK receptors share a common signaling motif in their cytoplasmic regions.

This canonical immunoreceptor tyrosine-based inhibitory motif (ITIM) is defined by the sequence (I/L/V/S)XYXX(L/V) (where X represents any amino acid, and slashes separate alternative amino acids that may occupy a given position). When ITIM-bearing receptors engage their ligands, the tyrosine residue is phosphorylated, probably by a Src family kinase, resulting in the recruitment of the lipid phosphatase SHIP-1—which degrades phosphatidylinositol-3,4,5-trisphosphate to phosphatidylinositol-3,4-bisphosphate—or the tyrosine phosphatases SHP-1 or SHP-2 (ref. 2). The inhibitory NK receptors recruit these phosphatases to the interface between the NK cell and its prospective target cell, and these tyrosine phosphatases suppress NK cell responses by dephosphorylating the protein substrates of the tyrosine kinases linked to activating NK receptors. By acting near the activating receptors, these phosphatases can terminate $Ca2^{+}$ influx, degranulation, cytokine production and proliferation of the NK cells.

Notably, these events are transient and spatially localized and do not interfere with the ability of the same NK cell to become activated upon encounter with a subsequent target cell lacking ligands for inhibitory NK receptors. The relative contribution of SHP-1, SHP-2 and SHIP to the inhibition mediated by the various ITIM-bearing NK receptors has not been clearly established. The inhibitory KIR, Ly49 and NKG2A receptors preferentially associate with SHP-1 or SHP-2 (ref. 2), whereas KLRG1 predominantly associates with SHIP3. Although the dephos-phorylation of many intracellular proteins has been documented after the engagement of the inhibitory NK receptors, the guanine nucleotide exchange factor Vav1, a downstream substrate modified by several different activating NK receptor signaling pathways (see below), seems to be a key substrate of SHP-1 recruited to the inhibitory KIRs4. Because inhibitory NK cell receptors regulate stimulation initiated by several activating NK receptors with quite distinct downstream signaling pathways, the precise substrates affected by these phosphatases may differ depending on the particular array of activating and inhibitory receptors that are brought into proximity when an NK cell encounters potential target cells. Studies in which SHP-1, SHP-2 or SHIP are selectively ablated in mature NK cells (to avoid effects on NK cell development) would be informative to determine the contribution of these different phosphatases in the regulation of the different activating NK receptors.

Finally, although the role of ITIMs in the inhibitory NK cell receptors is relatively well defined, the cytoplasmic domains of these molecules often contain other motifs that might regulate their activity. For example, the inhibitory molecule KIR3DL1 is constitutively phosphorylated on serine residues by protein kinase C, and this phosphorylation dampens the inhibitory function of KIR3DL1 and influences KIR3DL1 turnover and internalization5. These serine residues are conserved in several human and primate KIR proteins, suggesting that serine phosphorylation may play a general role in regulation of these inhibitory receptors. Therefore, a more careful analysis of the cytoplasmic domains of the NK cell inhibitory receptors is likely to reveal other mechanisms through which they are regulated.

Activating NK receptors

T and B cells possess a single antigen receptor that dominates their development and activation. Signals initiated through these antigen receptors are augmented by costimulatory molecules. In contrast, NK cells do not possess one dominant receptor, but instead rely on a vast combinatorial array of receptors to initiate effector functions. What constitutes an activating receptor or a costimulatory molecule in NK cells is uncertain and somewhat semantic. In a series of elegant experiments, Long and colleagues demonstrated that when agonist antibodies

are used to cross-link the 'activating' receptors on freshly isolated primary human NK cells, none of the receptors alone, with the exception of CD16, are able to elicit cytolytic activity or cytokine secretion6. However, when different pairs or combinations of receptors are simultaneously cross-linked, effector functions are triggered, with evidence in some cases for additive effects and in other cases synergistic actions. Therefore, Long suggested the term 'coactivating receptor' to describe the interplay and cooperation between these NK receptors. These observations suggest that a critical threshold of signaling that exceeds the counterbalancing influence of the inhibitory receptors must be achieved by these coactivating receptors in order for NK cells to mount a productive response.

Although mouse and human NK cells express numerous activating or coactivating NK receptors (Supplementary Table 2 online), many induce common signaling pathways and thus behave in a similar, if not identical, fashion. This review will focus on three biochemically distinct signaling pathways that have been identified in mouse and human NK cells: the ITAM-bearing NK receptor complexes, the DAP10-associated NKG2D receptor complexes and the CD244 receptor system.

ITAM-bearing NK receptor complexes

NK cells are unique among hematopoietic cells in that all mature NK cells constitutively express FcεRI-γ, CD3-ζ and DAP12 type I transmembrane-anchored proteins that exist as either disulfide-bonded homodimers or, in the case of FcεRI-γ and CD3-ζ, as disulfide-bonded heterodimers. All have minimal extracellular regions comprising only a few amino acids, principally the cysteine residues through which they dimerize. Most importantly, these proteins contain immunoreceptor tyrosine-based activation motifs (ITAMs), defined by the sequence (D/E)XXYXX(L/I)$X_{6–8}$YXX(L/I) (where $X_{6–8}$ denotes any 6 to 8 amino acids between the two YXX(L/I) elements) in their cytoplasmic domains. DAP12 and FcεRI-γ have a single ITAM, and CD3-ζ has three ITAMs per chain. There are no other known signaling motifs in the rather short cytoplasmic domains of these proteins, and mutation of the ITAM tyrosine residues abolishes their signaling function. Typically, the ITAM-bearing signaling subunits and their receptors assemble through oppositely charged amino acids in their transmembrane regions. The ITAM-bearing proteins all possess an aspartate residue within their transmembrane region. This charged residue is located centrally within the transmembrane of DAP12 but closer to the extracellular region in FcεRI-γ and CD3-ζ, and it is required for stable association with the associated receptors, which typically contain an oppositely charged amino acid (lysine or arginine) located in the same register within the transmembrane region (Fig. 1). One DAP12 homodimer pairs with each KIR2DS2 monomer or with each CD94-NKG2C heterodimer to form functional signaling complexes7. CD16 is an exception in that there are aspartate residues in the transmembrane domain of CD16 and in the associated FcεRI-γ and CD3-ζ subunits; however, these aspartate residues are required for assembly of the complex8,9. Further studies are needed to understand the basis for this interaction.

The signaling cascade downstream of the DAP12, FcεRI-γ and CD3-ζ subunits is, perhaps not unexpectedly, very similar to the well defined B and T cell antigen receptor signaling pathways. Engagement of these receptors causes the phosphorylation of the ITAM tyrosines, presumably by Src family kinases. NK cells express an abundance of Src family kinases, including Lck, Fyn, Src, Yes, Lyn and Fgr, and studies using gene-deficient mice suggest that their activities are probably redundant. Chemical inhibition of Src family kinase function blocks downstream signaling mediated by ITAM-coupled NK receptors. After phosphorylation, ITAM-bearing subunits bind the tyrosine kinases Syk and ZAP-70. In the mouse, Syk might be the predominant kinase10, whereas in humans both Syk and ZAP-70 seem to couple efficiently with these ITAM-bearing subunits (unpublished observations). Unexpectedly, NK cells still develop and have lytic activity in mice lacking both Syk and ZAP-70 (ref. 11) or in mice lacking

DAP12, FcεRI-γ and CD3-ζ (ref. 12), although their functions mediated through the ITAM-coupled receptors are impaired. ITAM-based signaling in NK cells lacking CD45, a membrane-anchored protein tyrosine phosphatase that regulates the enzymatic activity of Src family kinases, is impaired, but cytolytic activity initiated through the CD16–FcεRI-γ and Ly49D-DAP12 receptor complexes is largely intact, whereas transcription of cytokine genes is completely abrogated13–15. Therefore, the two major functions of NK cells—cytokine secretion and cytotoxicity—are regulated differentially downstream of ITAM-containing NK receptors.

The signaling pathways downstream of ITAM-containing NK receptors have been deduced from numerous studies from different laboratories that have studied signal transduction mediated by different DAP12-associated receptors or FcεRI-γ- and CD3-ζ-associated receptors (often CD16) in mouse and human NK cells (Fig. 2). Note that the different DAP12-, FcεRI-γ-, and CD3-ζ-associated NK receptor complexes might use different adapters or kinases in human and mouse NK cells or in NK cells at different stages of maturation or activation. For example, studies using gene-deficient mice have indicated that phospholipase C (PLC)-γ2 (ref. 16) and Vav2 and Vav3 (ref. 17) are predominantly used for ITAM-dependent signaling in mouse NK cells. In contrast, both PLC-γ1 and PLC-γ2 are phosphorylated in CD16-stimulated human NK cell lines18, and Vav1 is involved in CD16-mediated activation of human NK cells19.

ITAM-receptor activation induces actin cytoskeleton reorganization, which is required for cell polarization and release of the cytolytic granules containing perforin and granzymes, and results in the transcription of many cytokine and chemokine genes. After activation, the human CD16 receptor complex is polyubiquitinated and degraded in the lysosomes and proteasomes by a process that might involve the action of the c-Cbl E3 ubiquitin ligase20. Human CD16 receptor signaling is also negatively regulated by the recruitment of SHIP to the phosphorylated CD3-ζ subunits21. The fate of other stimulated ITAM-containing NK receptors has not been extensively investigated, and further studies should provide insights into how signaling through these receptors is regulated and terminated. It would also be useful to directly compare the signaling induced through a DAP12-associated receptor versus an FcεRI-γ- or CD3-ζ-associated receptor in the same NK cell clone to determine whether the downstream events are identical or have adaptor-specific properties.

NKG2D receptor complexes

A single gene with little polymorphism encodes the C-type lectin—like superfamily member NKG2D (ref. 22), which is a type II transmembrane-anchored glycoprotein expressed as a disulfide-bonded homodimer on the surface of NK cells, γδs T cells and $CD8^+$ T cells23,24. An arginine residue centrally located within the transmembrane region of NKG2D associates with the aspartate residue within the transmembrane domain of the DAP10 signaling subunit25 (Fig. 3). The extracellular region of DAP10, a 10 kDa protein expressed as a disulfide-bonded homodimer, contains the cysteine residues required for dimer formation, as well as serine residues that serve as sites for O-linked glycosylation25. The gene (*HSCT*) encoding DAP10 is located only a few nucleotides away from the gene (*TYROBP*) encoding DAP12 on chromosome 19 in humans or chromosome 7 in mice, and *HSCT* and *TYROBP* are arranged in opposite transcriptional orientations25.

In mice, NKG2D exists in long (NKG2D-L) and short (NKG2D-S) isoforms, which are generated by alternative RNA splicing26,27. Whereas NKG2D-L only pairs with DAP10, NKG2D-S can pair with either DAP10 or DAP12. The NKG2D-L protein is identical to the NKG2D-S protein except that NKG2D-L contains 13 more amino acids in its cytoplasmic domain, which prevent its association with DAP12. Resting mouse NK cells express very little

NKG2D-S, but NKG2D-S expression is induced after mouse NK cell activation *in vitro* or *in vivo*. An NKG2D-S isoform does not exist in humans, and human NKG2D can only associate with DAP10 (refs. 28,29). Domain mapping studies have established that the transmembrane region of human NKG2D can associate with DAP10 but not DAP12, whereas the transmembrane domain of mouse NKG2D promiscuously associates with either DAP10 or DAP12 (ref. 28).

The human NKG2D receptor complex is a hexamer, with one NKG2D homodimer associating with two DAP10 homodimers30. The composition of the mouse NKG2D-S—DAP12 complex, although not yet reported, is likely to be hexameric like other DAP12-containing receptor complexes7. DAP10 and DAP12 do not form disulfide-bonded heterodimers31; however, it has not been determined whether a single mouse NKG2D-S homodimer can form hexameric complexes with one DAP10 homodimer and one DAP12 homodimer.

Signals mediated through DAP10 are quite distinct from signals transmitted through ITAM-containing DAP12. The cytoplasmic domain of DAP10 is very small, with only 21 amino acids, and contains only one known signaling motif: the sequence YINM, which when phosphorylated is able to bind either the p85 subunit of phospha-tidylinositol-3-OH kinase (PI (3)K, through YXXM) or the adaptor Grb2 (throughYXNX) (Fig. 3). Because these two binding sites overlap, a single DAP10 chain will bind either p85 or Grb2, but not both. Mutagenesis studies have demonstrated that the tyrosine residue is essential for all signaling through DAP10, whereas mutation of the asparagine residue, which ablates Grb2 binding, or the methionine residue, which disrupts association with p85, results in different effects. Using chimeric receptors transfected into primary NK cells, Leibson and colleagues demonstrated that mutation of either the p85-binding site or the Grb2-binding site totally abrogates cytolytic activity initiated through DAP10 (ref. 32), consistent with studies demonstrating that PI3K inhibitors block NKG2D-mediated cytolysis33. Intact p85- and Grb-2-binding sites are also both required for optimal Ca^{2+} influx induced by cross-linking DAP10 (ref. 32). The downstream consequences of the recruitment of Grb2 versus p85 after DAP10 cross-linking, however, are distinct. A DAP10 construct containing a mutated p85-binding site induces phosphorylation of SLP-76 (SH2 domain—containing leukocyte protein of 76 kDa), Vav1 and PLC-γ2, whereas a DAP10 mutant unable to bind Grb2, but able to recruit p85, fails to induce phosphorylation of these substrates. Vav1 is upstream of SLP-76 and PLC-γ2 in this pathway32. In accordance with these *in vitro* studies with human NK cells, Vav1-deficient mice show impaired DAP10-dependent, NKG2D-mediated cytotoxicity, and Vav1-deficient NK cells are unable to effectively reorganize their actin cytoskeleton or polarize their microtubule organizing center toward target cells17,34,35. Recent data indicate that DAP10 binds to p85 and induces production of phosphatidylinositol-3,4,5-trisphosphate in the immune synapse formed between NK cells and NKG2D ligand—expressing target cells35. Interestingly, recruitment of Grb2 to the immune synapse is not prevented by mutation of the Grb2-binding site in DAP10, but it is prevented when p85 signaling is blocked. This suggests that Grb2 might be recruited to the immune synapse by DAP10 in two ways: directly, by binding to DAP10, and indirectly, possibly by recruitment of Sos1-Vav1-Grb2 complexes to phosphatidylinositol-3,4,5-trisphosphate—rich sites generated by activated PI3K at the immune synapse. Unlike the ITAM-containing receptors in NK cells, NKG2D-DAP10 receptor complexes do not require Syk family kinases or LAT (linker for activation of T cells) for signaling, as demonstrated by biochemical studies33 and by the ability of NK cells from mice lacking both Syk and ZAP-70, or LAT and NTAL (non—T cell activation linker), to mediate NKG2D-dependent cytolysis12,36.

The recruitment of Grb2 and p85 requires tyrosine phosphorylation of DAP10. PP2, a small-molecule chemical Src family kinase inhibitor, totally prevented signaling induced through the NKG2D-DAP10 complex33, suggesting that DAP10 might be phosphorylated by a Src family

kinase. Another candidate proposed for the phosphorylation of DAP10 is the kinase Jak3 (ref. 37). Recent data suggest that interleukin (IL)-15 stimulates Jak3-mediated phosphorylation of DAP10 and that this process is necessary to enable the NKG2D receptor to initiate killing of NKG2D ligand—bearing targets37. Long and colleagues6 had previously demonstrated the necessity for human NK cells to be 'primed' by stimulation with cytokines before certain NK cell receptors, including NKG2D, are competent to trigger cytotoxicity. Similarly, the ability of human $CD8^+$ T cells to kill when activated through the NKG2D receptor also requires previous stimulation by high concentrations of IL-2 or IL-15 (ref. 38). Further studies are needed to determine whethe signaling through the IL-2 and IL-15 receptor complexes uniquely enables NKG2D receptor function.

The events that occur downstream of PI3K and Grb2-Vav1 in the NKG2D signaling pathway in activated NK cells are less well characterized. In cultured human NK cell lines, stimulation with trimeric soluble recombinant NKG2D ligands (for example, ULBP-1 or ULBP-2) elicits phosphorylation of the kinases Jak2, Stat5, Akt, MEK1/2 and Erk, but not the kinases Jnk or p38 (ref. 39). PI3K inhibitors completely block MEK1/2 and Erk phosphorylation. How PI3K and/or the Grb2-Vav1 complex transduce NKG2D-DAP10 signals leading to activation of Jak2 and Stat2 or MEK1/2 and Erk is not yet known.

Although there is a consensus that engaging the NKG2D-DAP10 receptor complex on NK cells efficiently initiates cell-mediated cytotoxicity, the influence of NKG2D-DAP10 complexes on other effector functions, such as cytokine production, is less well understood. In the mouse, the situation is complicated by the presence in activated NK cells of both NKG2D-L—DAP10 and NKG2D-S—DAP12 complexes, as well as NKG2D-S—DAP10 complexes. As the extracellular domains of NKG2D-S and NKG2D-L are identical, it is impossible to preferentially engage these different complexes on wild-type NK cells. However, when NKG2D is cross-linked on activated NK cells from DAP10-deficient mice, which can only express NKG2D-S—DAP12 complexes, both cytotoxicity and cytokine secretion are induced27. In contrast, in DAP12-deficient mice, which only express NKG2D-DAP10 complexes, cytotoxicity but not cytokine production are induced26,27. In human NK cells, which only express NKG2D-DAP10 complexes, cross-linking of NKG2D triggers cytotoxicity but not cytokine secretion33. In contrast, human NK cells stimulated with soluble recombinant NKG2D ligands (MICA, ULBP-1, or ULBP-2) secrete cytokines, including interferon-γ, GM-CSF and MIP-1β29,40. In our own studies, we have observed that cytokines can be induced by cross-linking NKG2D on human NK cells, or on NK cells from DAP12-deficient mice, with monoclonal antibodies to NKG2D, provided that plates are coated with a sufficiently high concentration of the agonist antibody (unpublished observations). Therefore, it seems that although the mouse NKG2D-S—DAP12 complex more efficiently induces cytokine production, both human and mouse NKG2D-DAP10 complexes can induce cytokine production if cross-linked with a high-avidity ligand, at least *in vitro*.

Note that NKG2D signaling seems to proceed differently in NK cells and $CD8^+$ T cells. In humans and mice, $CD8^+$ T cells express DAP10 but usually do not express DAP12; therefore, in T cells, only NKG2D-DAP10 receptors are expressed. In humans, NKG2D-DAP10 receptor complexes are constitutively expressed on all resting and activated $CD8^+$ T cells23, whereas in mice $CD8^+$ T cells express NKG2D only after stimulation24,41. Cross-linking NKG2D alone on mouse or human $CD8^+$ cytotoxic T lymphocytes does not induce cytotoxicity23,41. However, when cultured for a prolonged time in high concentrations of IL-2 or IL-15, human CD8+ T cells acquire the ability to kill NKG2D ligand—bearing targets38,42. Cross-linking NKG2D on human $CD8^+$ T cells cultured in high concentrations of IL-2 or IL-15 induces phosphorylation of Erk and Jnk, but a PI(3)K inhibitor blocks only Erk phosphorylation38. Further studies are needed to explain the divergent behaviors of NKG2D in NK cells and T cells.

CD244

CD244 (originally named 2B4) is a member of the SLAM family of membrane receptors (reviewed in ref. 43), which are encoded by a cluster of genes on chromosome 1 (refs. 44, 45). Of the SLAM family members, human NK cells express CD244 (refs. 46,47), NTB-A (ref. 48) and CRACC (CD319)49, and mouse NK cells express CD244 (ref. 50) and CRACC (ref. 44). Essentially all immature and mature human and mouse NK cells express CD244. Initially, mouse CD244 was considered an activating receptor on NK cells51; however, subsequent studies suggested that in some circumstances CD244 might also inhibit NK cell effector functions52. In mice, two CD244 isoforms, designated long and short, are generated by alternative RNA splicing52; however, these distinct isoforms do not exist in humans and their functional properties in mice remain unclear. CD244 signal transduction involves interactions between SH2 domain—containing adaptor proteins and the tyrosine-based motif TIYXX(V/I) (referred to as an immunoreceptor tyrosine-based switch motif, ITSM) in the cytoplasmic domain of CD244. Three cytoplasmic SH2 domain—containing adaptor proteins recognize this motif: SAP (also known as SH2D1A or DSHP), EAT-2 (also known as SH2D1B) and ERT (also known as sh2d1c)44,45. Humans have functional genes encoding SAP and EAT-2, but ERT is expressed only in mice; its ortholog is a pseudogene in humans. Cross-linking CD244 causes tyrosine phosphorylation of the ITSMs and allows recruitment of either SAP or EAT-2 (or, in mice, also ERT), which bind to CD244 through their SH2 domains. SAP also binds to the Src kinase Fyn; an arginine residue at position 78 in SAP docks with an SH3 domain on Fyn (ref. 53;Fig. 4). EAT-2 and ERT lack this arginine residue, and it has been proposed that EAT-2 and ERT do not associate with Fyn54, although other studies suggest these adapters do interact with Fyn55,56. The ligand for CD244 is CD48, a cell surface glycoprotein expressed broadly on hematopoietic cells.

Veillette and colleagues proposed that CD244-SAP-Fyn receptor complexes activate NK cells, whereas CD244-EAT2 and CD244-ERT receptor complexes inhibit NK cell activation44,45. This hypothesis is supported by the finding that NK cells from patients with a loss-of-function mutation in the *SH2D1A* (SAP) gene, a genetic disorder designated X-linked lymphoproliferative disease (XLP), are unable to kill CD48-bearing targets57–59. Patients with XLP present with a lymphoproliferative disorder initiated by infection with Epstein-Barr virus (EBV) and often succumb to EBV-induced malignancies. In the absence of SAP, cross-linking CD244 on NK cells either fails to induce cytotoxicity or, in some cases, inhibits NK cell—mediated lysis. Why in some situations cross-linking of CD244 in the absence of SAP causes no activation of human NK cells and in other cases causes inhibition has not been explained. Perhaps with no adaptor CD244 is simply nonfunctional, whereas in NK cells expressing EAT2 without SAP the CD244-EAT2 complex is inhibitory. The latter possibility is consistent with the observation that NK cells in EAT2-deficient or ERT-deficient mice demonstrate augmented cytolytic activity and interferon-γ secretion when stimulated through a variety of activating receptors, including CD16, Ly49D and NKG2D54. Tyrosine residues in the C-terminal region of EAT2 and ERT are phosphorylated in NK cells, and mutation of these tyrosines to phenylalanine abrogates the inhibitory function of these adaptors54. Also consistent with the hypothesis that CD244-SAP-Fyn functions as an activating NK cell receptor, SAP-deficient or Fyn-deficient mice have impaired NK cell—mediated killing of CD48-bearing targets *in vitro* and *in vivo*60. Although the model proposed by Veillette and colleagues to explain the divergent functions of CD244 is well supported by evidence, including functional studies in the SAP-and EAT-2-deficient animal models, a recent study has reported that Fyn can interact with either SAP or EAT-2, questioning the conclusion that association of EAT-2 with CD244 results in exclusively inhibitory function by this receptor complex56.

Studies of CD244-deficient mice also suggest that the situation may be more complicated. NK cells from CD244-deficient mice demonstrate enhanced cytotoxicity and cytokine production when challenged with CD48-bearing tumor targets *in vitro* and *in vivo*61. Curiously, CD244-deficient mice more efficiently reject B16 melanoma cell lines expressing CD48, but this phenomenon is only observed in male mice62. In addition, NK cells from CD244-deficient mice kill NK cells from syngeneic wild-type mice, implying that interactions between inhibitory CD244 receptors and CD48, both expressed on NK cells, prevent fratricide63. These results suggest that the inhibitory form of CD244 is predominant on mouse NK cells. Notably, if activated NK cells mediate fratricide, this might explain the seemingly paradoxical findings that, in other situations, CD244-deficient mice show impaired, rather than enhanced, NK cell responses64.

The predominant CD244 receptor expressed on mature human NK cells is clearly activating or coactivating. Transfecting certain mouse cell lines with human CD48 renders them more sensitive to lysis by human NK cells47, and antibodies neutralizing CD244 or CD48 partially block the killing of target cells expressing CD48 (ref. 65). The activating function of CD244 is abrogated in subjects with XLP57–59, demonstrating that the CD244-SAP receptor complex is an activating receptor in human NK cells. The predominance of CD244 inhibition in mice and activation in humans might be explained by the relative abundance of SAP, EAT2 and ERT in mouse versus human NK cells, but this possibility has not yet been addressed experimentally.

The cytoplasmic domain of human CD244 contains three prototypical ITSMs that when phosphorylated can bind SAP, and a TLYSLI sequence that when phosphorylated can interact, at least *in vitro*, with SHIP, SHP-1, SHP-2 and Csk66. One model proposed to explain the paradoxical functions of CD244 in different NK cell populations hypothesized that SAP can competitively displace SHIP, SHP-1 or SHP-2 and thereby prevent the suppressive activity of these phosphatases and promote NK cell activation. According to this model, in the absence of SAP (for example, in patients with XLP), inhibition would dominate because these phosphatases would have unobstructed access to the phosphorylated TLYSLI motif in CD244. Although data from *in vitro* experiments in which these phosphatases were overexpressed are consistent with this model47, interactions of CD244 with SHIP, SHP-1, SHP-2 and Csk have not been validated in a physiological context using NK cells and CD48-bearing targets. Moreover, the apparent binding affinities of the tyrosine motifs in the cytoplasmic domain of CD244 for the SH2 domains of SHP-1, SHP-2 and SHIP are relatively weak and lower than those for the SH2 domains of SAP or EAT-2 (ref. 56). Therefore, although an inhibitory role for CD244 has been clearly demonstrated in mouse NK cells and, in relatively infrequent situations, in human NK cells, the molecular basis of this inhibition has not been established.

Cross-linking CD244 on human NK cells induces tyrosine phosphorylation of many substrates, including CD244, Vav1, SHIP-1, c-Cbl, 3BP2, p38 MAPK, ERK1/2 and PLC-γ, although different substrates have been identified by different laboratories67–72. Both mouse and human CD244 have been reported to associate with LAT in some studies71,73,74 but not others67. Consistent findings indicate that cross-linking CD244 on human NK cells induces phosphorylation of CD244, Vav1, SHIP and c-Cbl; however, other aspects of the signaling pathway downstream of CD244 vary considerably depending on the experimental conditions or cell type used. As discussed previously, this may reflect the relative abundance of SAP, EAT2 and ERT, as well as the activation status of the NK cells analyzed.

CD244-induced activation of human NK cells is subject to negative regulation by the inhibitory KIR molecules when target cells coexpress both CD48 and a relevant MHC class I ligand68, 75. In this situation, KIRs block tyrosine phosphorylation of CD244 and also prevent actin-dependent reorientation of CD244 in the cell membrane. KIR-mediated inhibition of CD244

signaling is likely to be mediated by SHP-1, which is the predominant phosphatase recruited by inhibitory KIRs. Interactions between mouse CD244 and SHP-1 were recently revealed by studies of NK cells in SHIP-deficient mice. NK cells from SHIP-deficient mice express higher amounts of inhibitory CD244 receptors than wild-type NK cells and thus show diminished NK cell effector function against CD48-bearing targets presenting ligands for the activating Ly49H or NKG2D receptors76. The enhanced inhibition of SHIP-deficient NK cells is explained by the higher amounts of SHP-1 recruited to the cytoplasmic domain of CD244 in these cells77. Therefore, the net contribution of CD244 to NK cell signaling in humans and mice is tightly regulated by the interplay between inhibiting and activating receptors and can be influenced by the density at which these receptors and their ligands are expressed on NK cells and on their targets, respectively.

Concluding remarks

Here I have reviewed the structural and biochemical features of selected activating and inhibitory receptors that have been identified on human and mouse NK cells. These receptors are not static, but are regulated in a spatial and temporal fashion. Similarly to a T cell, a NK cell forms a synapse with the target cell where adhesion molecules (for example, LFA-1) and the activating or coactivating NK cell receptors interact with their ligands on target cells. If the target cells express MHC and non-MHC class I ligands for NK inhibitory receptors, phosphatases recruited by the inhibitory receptors are brought to the synapse, where they act to dampen or prevent NK cell activation. The synapses between NK cells and their targets are brief, allowing the NK cell to detach and engage other potential target cells78. Anintriguing question deserving more study is how NK cell contact with targets is terminated. Interactions between an NK cell and a potential target cell that is spared because it expresses sufficient ligands for the inhibitory NK cell receptors do not impair the ability of the same NK cell to productively engage other targets. Now that most (but probably not all) of the inhibitory and activating NK cell receptors have been identified, future studies will be directed toward understanding the dynamic interactions between these molecules.

Reductionist approaches have proven instrumental in identifying most of the activating and inhibitory NK cell receptors, their ligands, and their signaling pathways. However, this strategy fails to recapitulate the real-life situation in which NK cells interact with normal or abnormal host cells in environments containing soluble mediators that enhance (for example, type I interferons, IL-12, IL-15, IL-18) or suppress (for example, TGF-β) NK cell responses. Systems biology approaches will be needed to precisely understand how these signals are processed intracellularly and regulate the behavior of NK cells in health and disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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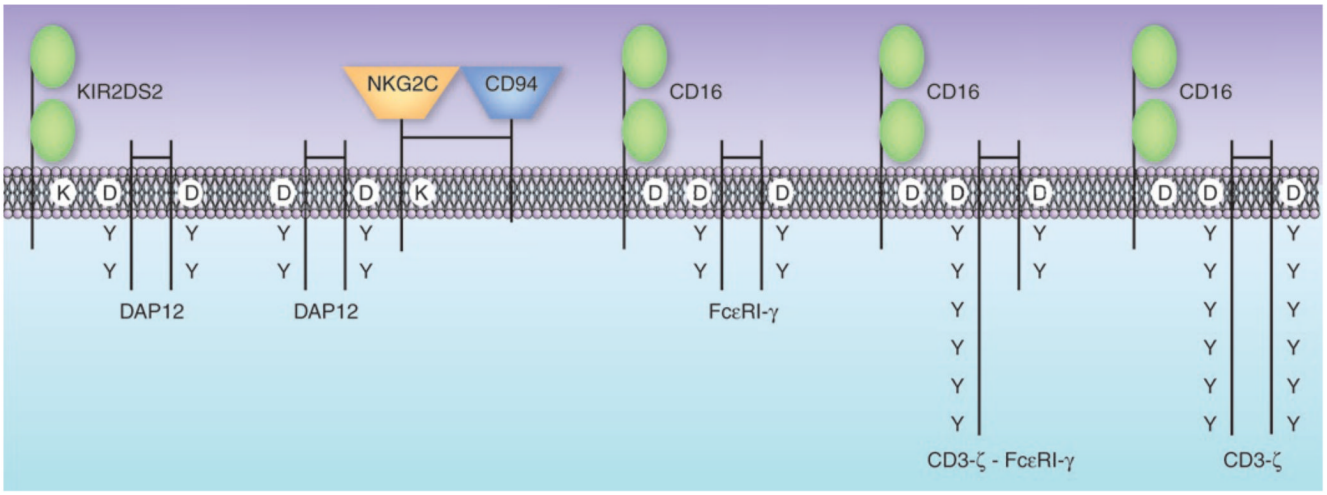

Figure 1.

ITAM-containing NK receptors. Schematic representation of NK receptors of the immunoglobulin superfamily or C-type lectin—like family that pair with the ITAM-bearing DAP12, FcεRI-γ and CD3-ζ signaling subunits. For a comprehensive list of ITAM-signaling NK cell receptors, see Supplementary Table 1. Note that human CD16 can pair with homodimers of FcεRI-γ or CD3-ζ or with heterodimers of FcεRI-γ and CD3-ζ, whereas mouse CD16 signals efficiently only with homodimers of FcεRI-γ. ITAM-bearing signaling subunits contain aspartate residues (D) within their transmembrane segments that associate noncovalently with oppositely charged lysine or arginine residues within the transmembrane of the receptors, an exception being CD16, which also has an aspartate residue within its transmembrane. Y, tyrosine residues within ITAM domains.

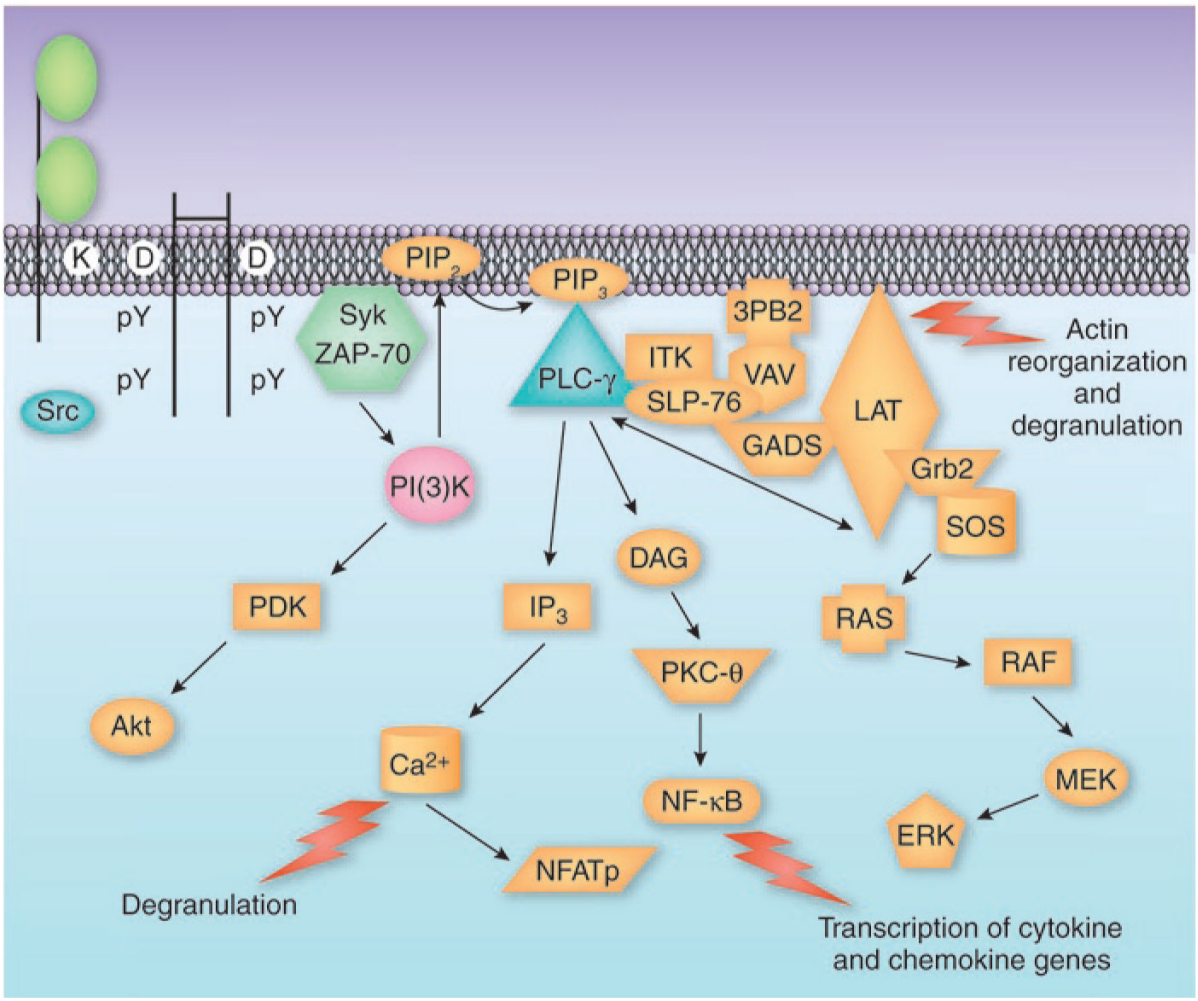

Figure 2.

ITAM-mediated signaling in NK cells. ITAM-bearing signaling subunits are phosphorylated, probably by Src family kinases, after receptor engagement. Syk and/or ZAP-70 (both of which are expressed by human and mouse NK cells) are recruited to the phosphorylated ITAMs, initiating a cascade of downstream signaling as depicted. The signaling pathways depicted are hypothetical and were deduced by synthesizing results from many studies investigating ITAM-coupled receptor signaling in human and mouse NK cells. DAG, diacylglycerol; IP_3, inositol-3,4,5-trisphosphate; PIP_2, phosphatidylinositol-3,4-bisphosphate; PIP_3, phosphatidylinositol-3,4,5-trisphosphate; pY, phosphotyrosine; ITK, tyrosine kinase; GADS and 3BP2, adaptor proteins; NFATp and NF-κB, transcription factors; PDK, phosphoinositide-dependent protein kinase; PKC-θ, protein kinase C-θ; RAF, mitogen-activated protein (MAP) kinase kinase kinase; RAS, GTPase.

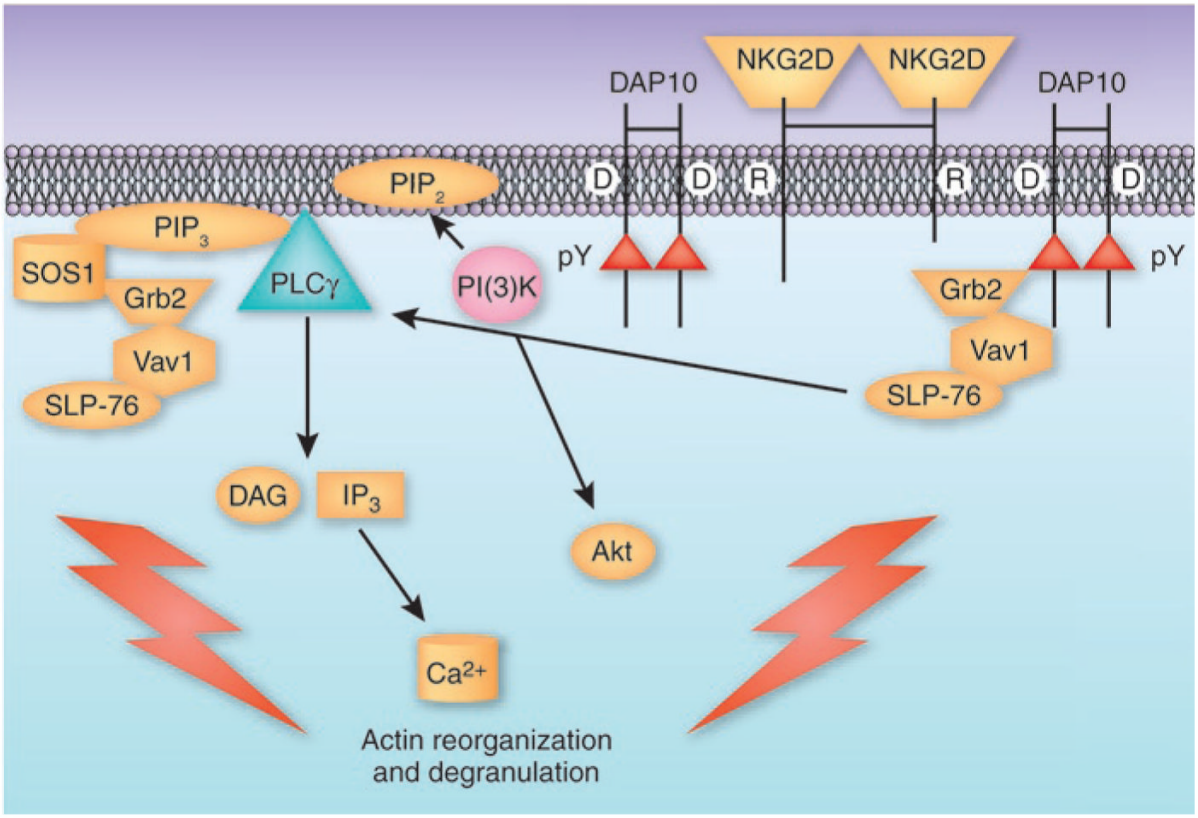

Figure 3.

DAP10-mediated signaling in NK cells. Cross-linking NKG2D causes NK cell activation that involves the recruitment of the p85 subunit of PI(3)K and recruitment of the Grb2-Vav1-Sos1 complex to the phosphorylated YINM motif in the cytoplasmic domain of DAP10. These events trigger distal signaling cascades as depicted.

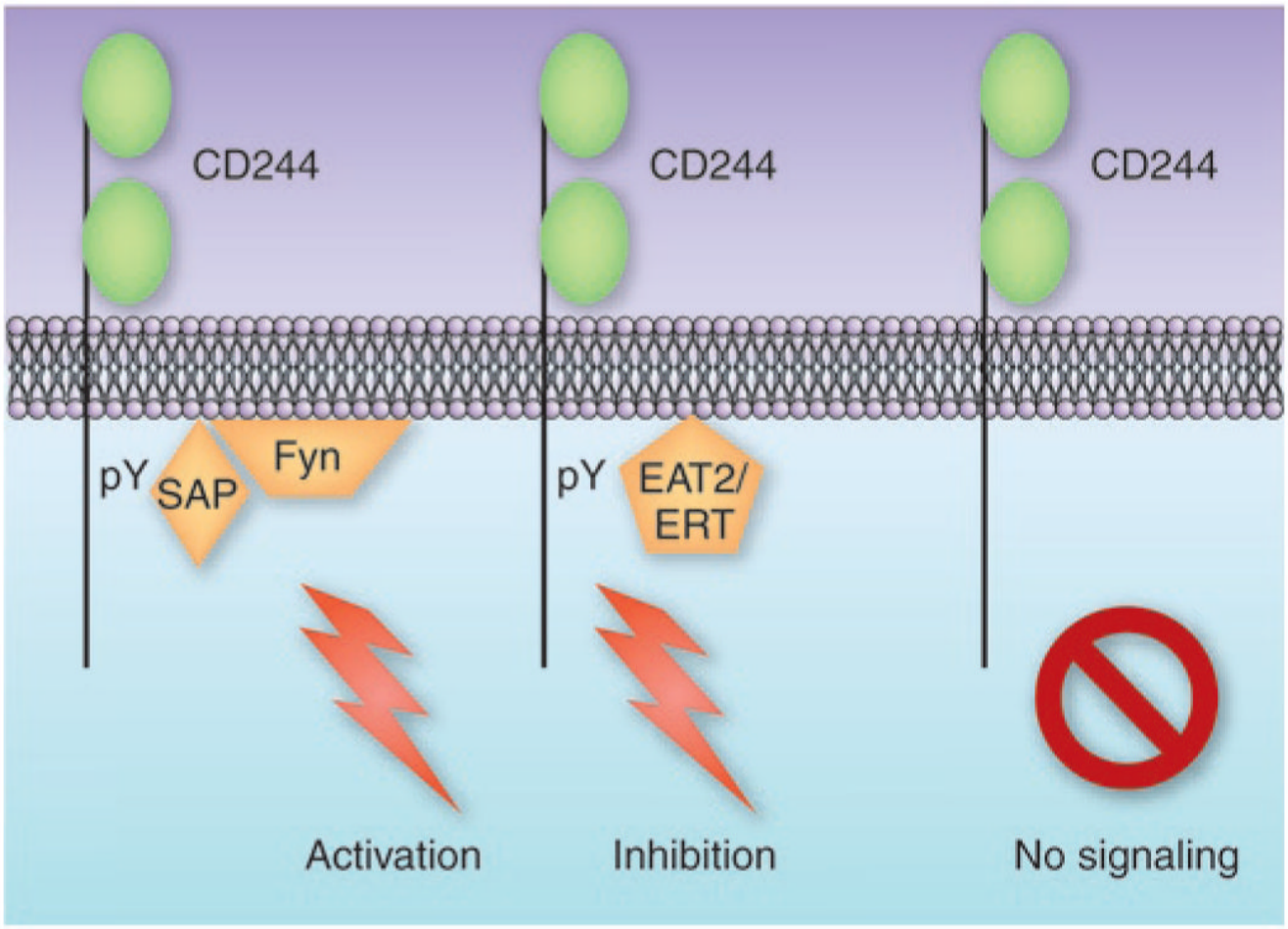

Figure 4.
CD244 receptor complexes in NK cells. Phosphorylation of the tyrosines in the TIYXX(V/I) motifs in the cytoplasmic domain of CD244 can recruit the adaptor proteins SAP, EAT2 or ERT (ERT exists in mice, but not humans). SAP binds to Fyn to mediate signal transduction. It has been proposed that the CD244-SAP–Fyn complex is responsible for NK cell activation when NK cells encounter target cells expressing CD48, a ligand of CD244. Alternatively, evidence suggests that CD244-EAT2 and CD244-ERT complexes deliver inhibitory signals into NK cells, although this remains controversial44,45,56,61.