Natural killer cell receptor signaling
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Natural killer (NK) cell immune responses are regulated by a balance of activating and inhibitory signals transmitted by cell surface receptors. Immunoreceptor tyrosine-based inhibition motifs in the cytoplasmic domains of inhibitory NK receptors recruit tyrosine or lipid phosphatases, which modulate the activation signals transmitted by receptors linked to the Syk and ZAP70 tyrosine kinases and phosphatidylinositol-3 kinases. In addition, recent studies of gene-deficient animals, in particular Syk and ZAP70 double-deficient mice, suggest that NK cells possess a robust and potentially redundant receptor system to ensure their development and function.

Introduction
The observation that natural killer (NK) cells preferentially kill certain cells if they lack the expression of MHC class I predicted the existence of inhibitory receptors that regulate NK cell activation. Activation of NK cells in the absence of MHC class I on the target cell was considered to occur by ‘default’, resulting in the killing of any cell lacking MHC class I. However, this notion failed to consider the fact that NK cells do not kill erythrocytes, which in humans do not express MHC class I, and they rarely kill normal resting cells even if they express only low levels of MHC class I. Furthermore, the ‘killing by default’ concept couldn’t explain how an NK cell could attack something that it couldn’t recognize in a positive fashion. The recent discovery of a plethora of activating NK receptors helps to resolve the question of how NK cells recognize potential targets. In addition, it has become apparent that the engagement of inhibitory receptors for MHC class I does not globally suppress NK cell activation, but rather a balance between stimulation and inhibition dictates the nature of the immune response.

NK receptors and signaling pathways

NK receptors associated with ITAM-bearing transmembrane adaptor proteins
Perhaps not surprisingly, the positive and negative signaling pathways used by NK cells share many common features with the immune receptors expressed on B and T lymphocytes. Although numerous NK cell receptors have been identified, these converge on a few biochemical pathways employed by most leukocytes (reviewed in [1–4]). In particular, the activating NK cell receptors that are best characterized use signaling elements also employed by the B- and T-cell antigen receptors; there is a division of labor, with ligand recognition and signal transduction delegated to independent protein subunits and assembled into the functional receptor complex.

Signals are transmitted by small transmembrane-anchored adaptor proteins that possess immunoreceptor tyrosine-based activation motifs (ITAMs) in their cytoplasmic domains. NK cells express the ITAM-bearing CD3ζ, FcεRIγ and DAP12 adaptor proteins. CD3ζ and FcεRIγ can be expressed as disulfide-bonded homodimers or disulfide-bonded heterodimers, whereas DAP12 is exclusively expressed as a disulfide-bonded homodimer. Associations between these ITAM adapters and their receptors are predominantly mediated by interactions within their transmembrane regions, often involving pairs of oppositely charged amino acids that form stable salt bridges. Numerous NK receptors have been identified that pair with DAP12 (e.g. in mice several activating Ly49 receptors, CD94/NKG2C and CD94/NKG2E, and in humans several activating killer cell immunoglobulin-like receptors [KIRs], CD94/NKG2C and NKp44; Figure 1), CD3ζ or FcεRIγ (e.g. NKR–P1C and CD16 in mice, and NKp30, NKp46 and CD16 in humans; Figure 2). Interestingly, in mice CD16 is unable to pair with CD3ζ, but uses FcεRIγ. A recent study by Arase et al. [5] reported that heterodimers of CD3ζ and FcεRIγ...
compete with FcεRIγ homodimers and negatively regulate CD16-mediated activation of NK cells.

Ligation of any ITAM-bearing receptor complexes results in the recruitment and activation of the tyrosine kinases Syk and ZAP70, both of which are expressed by all NK cells. The most extensively studied NK receptor is CD16, a low-affinity IgG receptor responsible for antibody-dependent cellular cytotoxicity (ADCC). CD16 signaling in NK cells, initiated by either CD3ζ or FcεRIγ, is quite similar to TCR-induced signal transduction in T cells; phosphorylation of the ITAMs is probably mediated by a src family kinase, thereby facilitating recruitment of Syk and ZAP70. Downstream events include the phosphorylation of SLP-76, 3BP2 [6], Shc, [7] p85 PI3-kinase, c-Cbl, phospholipase C (PLC)-γ1 and PLC-γ2, the mobilization of Grb2, linker for the activation of T cells (LAT), Vav-1 and Vav-2, the elevation of intracellular Ca2⁺ levels, and the activation of Rho, Ras, p38 mitogen activated protein kinase (MAPK) and extracellular signal-regulated kinase (ERK).

Signaling via CD16 activates nuclear factor of activated T cells (NFAT) and results in the production of cytokines.
including IFN-γ, GM-CSF and several chemokines, and causes degranulation of NK cells. Similar to TCR signaling, CD16 activation can cause apoptosis in IL-2-activated NK cells. The biochemical events accompanying stimulation of other ITAM-based NK receptors are not well characterized, but will probably be similar to CD16-induced activation. As with CD3ζ or FceRIγ, stimulation of NK cells through DAP12 activates Syk, and ZAP70 and initiates NK cell-mediated cytotoxicity and cytokine production. Ortaldo and colleagues [9] used microarray analysis to explore gene transcription induced by activation through the Ly49D–DAP12 receptor complex in murine NK cells, revealing genes that are either increased or decreased in expression. Of interest, the most potently induced genes were certain chemokines, such as macrophage-inflammatory protein-1α or -1β (MIP-1α, MIP-1β), that might be involved in recruitment of other leukocytes to sites of infection.

The activating NKG2D receptor complex

The NKG2D receptor has received considerable attention as it allows NK cells to recognize virus-infected and transformed cells that have upregulated expression of the NKG2D ligands (RAE-1 and H-60 in mice, and MICA, MICB and ULBP in humans; reviewed in [1–3]). Expression of NKG2D on the cell surface requires its association with DAP10, a transmembrane-anchored adaptor protein expressed as a disulfide-linked homodimer. The short cytoplasmic domain of DAP10 contains a YxxM motif (in amino acid one-letter code, where x denotes any amino acid) that binds to the p85 subunit of phosphatidylinositol-3 kinase (PI3 kinase) and Grb2 upon phosphorylation. Although the ‘long’ NKG2D (NKG2D-L) glycoprotein associates exclusively with DAP10, recent studies by Diefenbach and colleagues [9**] have identified a ‘short’ (NKG2D-S) alternatively spliced isoform of mouse NKG2D that is able to associate with both DAP10 and DAP12 (Figure 3). NKG2D-S was also observed in NK cells obtained from DAP10-deficient mice [10**]. Resting NK cells express only NKG2D-L; however, after activation by IL-2 in vitro or by poly-I:C (an inducer of type I interferons) in vivo, NK cells also transcribe NKG2D-S [9**]. Therefore, in activated NK cells the NKG2D receptor stimulates both ITAM-based and PI3 kinase-associated pathways, providing stimulation and co-stimulation by the same receptor.

NKG2D is also expressed in activated mouse CD8+ T cells, which also express DAP10 but not usually DAP12 [11*]. Consequently, in T cells, NKG2D is unable to stimulate ZAP70 or Syk and can only provide co-stimulation for TCR-induced T-cell activation. The downstream events accompanying NKG2D stimulation have not been extensively characterized, but may be complicated to dissect if DAP10, DAP12 and potentially other molecules are involved, depending on the particular cell type and their activation state. Interestingly, Sutherland et al. [12*] observed that stimulation of human NK cells with soluble NKG2D ligands resulted in the activation of Janus kinase 2, STAT5, ERK, MAPK and PI3 kinase/Akt signal transduction pathways. Although activation of PI3 kinase/Akt is explained by the presence of the YxxM motif in DAP10, the mechanism by which the other signaling pathways are recruited requires further investigation. In our own studies of NKG2D signaling in human NK cells, we have observed activation of PI3 kinase and Akt, but not phosphorylation of Syk, ZAP70 or ERK (A Zingoni, L Lanier, unpublished data). However, as noted above, NKG2D may be hardwired to different activation pathways in different cells.

The paradigm of stimulation of T cells through a dominant TCR and co-stimulation through secondary receptors that amplify the TCR signal may not apply to NK cells.
NK receptor signaling not involving ITAM-based or DAP10 pathways

NK receptors not using ITAM adapters or DAP10 have also been defined. The CD244 (2B4) and NTB-A [13] receptors contain TyxxV/I motifs in their cytoplasmic domains, which permit association with the cytoplasmic SLAM-associated protein (SAP) adaptor protein (also called SH2 domain-containing protein 1A; SH2D1A; reviewed in [14]). Upon activation, CD244 associates with LAT and localizes to lipid rafts, a critical interaction for CD244-mediated signal transduction [15,16]. SAP is of interest because a loss-of-function SAP gene mutation in human X-linked lymphoproliferation disease results in life-threatening infections with Epstein-Barr virus (EBV) and EBV-associated B-cell malignancies. SAP and its associated receptors is reviewed in greater depth in another article in this section [17].

CD160 is an activating NK receptor that recognizes certain HLA class I molecules. Similar to other glycosylphosphatidylinositol (GPI)-anchored proteins, CD160 probably resides in lipid rafts and might thus associate with kinases localized in these domains to achieve signaling [18].

Although not restricted to NK cells, integrins (in particular, lymphocyte function-associated antigen [LFA]-1; also called CD11a/CD18) have been implicated in NK cell activation and effector function. In a recent study by Barber and Long [19], human NK cells adhere to insect cells transfected with human intercellular adhesion molecule 1 (ICAM-1, CD54) and this is greatly enhanced in the presence of IL-2 or IL-15. In addition, if the insect cells co-express CD58 or CD48, (ligands for the CD2 and CD244 [2B4] receptors, respectively) adhesion is enhanced. Inhibitors of src kinases or PI3 kinase prevented CD244-mediated signal transduction [15,16]. SAP is of interest because a loss-of-function SAP gene mutation in human X-linked lymphoproliferation disease results in life-threatening infections with Epstein-Barr virus (EBV) and EBV-associated B-cell malignancies. SAP and its associated receptors is reviewed in greater depth in another article in this section [17].

Inhibitory NK receptor signaling

Paradoxically, the mechanism of the inhibitory NK cell receptors was resolved long before an understanding of the activation receptors became apparent (extensively reviewed in [20]). All of the inhibitory KIRs and Ly49 receptors possess immunoreceptor tyrosine-based inhibition motifs (ITIMs) in their cytoplasmic domains. Upon engaging a MHC class I ligand, these ITIMs are phosphorylated and recruit phosphatases to counteract cellular activation. The predominant phosphatases associated with KIR and Ly49 are Src homology (SH)-containing tyrosine phosphatase (SHP)-1 and SHP-2. However, Wang et al. [21] have recently shown an association of inhibitory Ly49 receptors with the lipid phosphatase SH2 domain-containing inositol-5 phosphatase (SHIP). The substrates of these phosphatases are still under active investigation, but they might depend upon which activating receptors are being modulated. A critical central substrate of SHP-1 may be Vav, as revealed by substrate-trapping experiments using a catalytically inactive KIR-SHP-1 chimeric receptor (EO Long, personal communication).

One of the human KIR molecules, KIR2DL4 (CD158d) is schizophrenic, containing an ITIM in the cytoplasmic domain but also exhibiting activating functions [22,23, 24]. When introduced into a chimeric receptor, the KIR2DL4 cytoplasmic domain inhibits NK cell activation. By contrast, cross-linking the endogenous KIR2DL4 on NK cells can activate cell-mediated cytotoxicity and cytokine production. A positively charged amino acid in the transmembrane of KIR2DL4 suggests association with an adaptor protein, but this receptor apparently does not pair with any of the usual suspects (DAP12, DAP10, CD3ζ or FceRIγ). The physiological function and ligand of this receptor, when identified, should prove interesting.

Spatial organization of NK receptors on the membrane

Now that many of the NK receptors have been identified, attention has turned to their spatial localization in the membrane and their movement upon encountering target cells expressing relevant ligands. Several laboratories have investigated the localization of receptors and signaling molecules during encounters between NK cells and target cells [25–29]. There is a consensus that NK cell-mediated cytotoxicity requires adhesion to target cells, polarization of the relevant receptors and exocytosis of perforin-containing granules at the interface.

When an appropriate HLA class I ligand for an inhibitory NK receptor is present on the target cells, the inhibitory receptors are redistributed to the interface between NK cells and target cells and killing may be diminished. Vyas and colleagues [27,28] reported that when human NK cells encounter NK-sensitive targets there is a localization of Lck, protein kinase C0, PLC-γ1, Itk, Syk, ZAP70 and SLP-76 to the intercellular interface, together with the polarization of talin (the microtubule organizing center) and lysozymes. SHP-1 was also found to localize to the cellular interface; however, in the presence of a ligand for an inhibitory KIR on the target cell, SHP-1 entered the central zone of the interface but was only seen at the periphery of the region upon encounters with NK-sensitive targets [27,28]. Previous studies demonstrated that chemical inhibitors of the src and Syk family kinases prevented target cell-induced lipid raft formation in NK cells and expression of a dominant-negative SHP-1 prevented the activity of an inhibitory KIR in disruption of lipid rafts at the NK cell and target interface. The generation of functional lipid rafts at the interface of NK
cells and target cells required an intact RhoA-ROCK-LIMK1 pathway to achieve the necessary regulation of the actin cytoskeleton [25].

Watzl and Long [30] examined the localization of CD244 and concluded that it is phosphorylated and recruited into lipid rafts when NK cells encounter NK-sensitive targets expressing CD48. Co-engagement of CD244 and an inhibitory KIR prevents actin-dependent mobilization of CD244 and its subsequent phosphorylation. Collectively, these studies are beginning to reveal the complex interplay of signaling molecules involved in NK cell activation.

**NK receptor signaling: development and effector function**

The development of NK cells is remarkably robust and resilient to disruption. Although some effector functions are affected, normal numbers of NK cells are present in mice lacking DAP12, CD3ζ and FcRIγ, individually or in combination. Furthermore, the ability of NK cells to develop in the absence of ITAM-mediated signaling has been confirmed by examining mouse NK cells lacking both Syk and ZAP70. NK cells from mice lacking both Syk and ZAP70 killed NK-sensitive tumors such as Yac-1 and RMA-S at levels comparable to wild-type mice, indicating that non-ITAM-based signaling pathways are responsible for this function [31**]. As anticipated, however, ADCC through CD16 was absent in mice lacking either FcRIγ or both Syk and ZAP70. As NK cells express both Syk and ZAP70, these kinases appear to have a redundant function in mice lacking either kinase alone. As expected, the activating Ly49D receptor was non-functional in mice lacking DAP12 or both Syk and ZAP70 [31**]. DAP12-deficient mice on the C57BL/6 background are rendered more susceptible to mouse cytomegalovirus (MCMV) infection [32] because Lyn49H, an activating receptor for the m157 MCMV glycoprotein, is non-functional [33**]. Although the mouse NKR-P1C receptor associates with FcRIγ, this receptor retains function in Syk and ZAP70 double-deficient mice, suggesting that an alternative signaling pathway is used by this receptor [31**]. As B- and T-cell development are disrupted in mice lacking Syk and ZAP70, it is quite surprising that NK cell development is intact, with loss of only selective effector functions.

NK cells also develop normally in mice lacking DAP10 [10**]. In resting NK cells, NKG2D-mediated functions were severely compromised; upon activation, however, NKG2D activity was partially restored by the association of NKG2D-S with DAP12. NKG2D receptor function in NK cells does not depend on the presence of DAP12 because NK cells from DAP12-deficient mice and Syk and ZAP70 double-deficient mice retained the ability to kill NKG2D ligand-bearing tumors (F Colucci, personal communication; [33**]). Normal numbers of NK cells are present in mice lacking Lck [34], Fyn [34], SLP-76, LAT, SHP-1 and SHIP [21]. Fyn-deficient [34] or SHIP-deficient [21] mice have NK cells, although the repertoire of inhibitory Ly49 receptors is affected in these animals. NK cells also develop normally in mice lacking Vav-1, although interestingly they have diminished cytolytic activity against several tumor targets [35**,36**], but retain the ability to make cytokines [36**]. These findings not only implicate Vav-1 as critical in NK cell-mediated cytotoxicity, but also dissociate the functions of NK cell degranulation and cytokine production. NK cells develop normally in LAT-deficient mice [15], possibly because they express the related adaptor protein non-T-cell activation linker (NTAL; [37]), but lack CD244-mediated signaling.

As yet, the only defined signaling pathway absolutely required for NK cell development involves their ability to respond to IL-15. Disruption of the genes encoding IL-15, IL-15α receptor, IL-2 receptor β and IL-2 receptor common γ chain, or Jak-3 (all required for response to IL-15) completely abrogated NK cell development, whereas only rather modest affects on NK cell development were caused by disruption of IL-2, IL-4, IL-7, or IL-9.

Analysis of NK cells in certain transcription factor gene-deficient mice have revealed impairments in NK development or function (reviewed in [38*]). Numerous studies have evaluated the signaling requirement of ‘natural cytotoxicity’ by using pharmacological inhibitors or transfection of dominant-negative signaling molecules (reviewed in [4*]). On the basis of these studies, Djeu and colleagues [38*] proposed a model of human NK cell-mediated cytotoxicity involving a Syk-P13 kinase-Rac 1-PAK1-MEK1/2-ERK1/2 pathway that is independent of Ras activation. Although quite compelling, the major limitation of these and other studies of ‘natural cytotoxicity’ is the lack of identification of the particular NK cell receptors that are involved in the process.

**Conclusions**

We are beginning to understand NK cell recognition and signal transduction in molecular detail. Although many NK receptors have been discovered in the past few years, research into understanding their biological relevance and the regulation between positive and negative signaling will be areas of intense study in the future. The ITAM-based receptors, integrins and NKG2D can now explain many of the phenomena referred to as ‘natural killing’; however, the ability of NK cells from Syk and ZAP70 double-deficient mice to recognize and respond to tumors lacking ligands for NKG2D still leaves open the question of which receptors participate in this activity. Nonetheless, recent progress makes it clear that NK cells do not rely on one omnipresent receptor for their immune functions, rather they are more flexible in their ability to select from many receptors to accomplish their tasks.
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References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:
• of special interest
•• of outstanding interest
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24. Rajagopalan S, Fu J, Long EO: Cutting edge: induction of IFN-γ production but not cytotoxicity by the killer cell Ig-like receptor kird4d (cd158d) in resting NK cells. J Immunol 2001, 167:1877-1881. The authors suggest that the induction of cytotoxicity and cytokine production may be dissociated when NK cells are activated through an unusual KIR.


Surprising findings that normal numbers of NK cells develop in mice lacking both Syk and ZAP70 and many effector functions are intact.


A formal demonstration that a DAP12-associated receptor is involved in vivo in immunity to a viral infection.


A demonstration that a DAP12-associated receptor Ly49H recognizing a viral glycoprotein ligand fails to function in DAP12-deficient mice.


These two papers are demonstrations that Vav-1 is important in NK cell-mediated cytotoxicity against certain tumors.


See annotation to [35].


The authors propose a testable biochemical model to explain ‘natural cytotoxicity’, implicating a Syk-Pi3 kinase-Rac 1-PAK1-MeK1/2-ERK1/2 pathway.