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Activation, co–activation, and co–stimulation of resting human NK cells

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Abstract

NK cells possess potent perforin– and IFN-γ–dependent effector functions that are tightly regulated. Inhibitory receptors for MHC class I display variegated expression among NK cells, which confers specificity to individual NK cells. Specificity is also provided by engagement of an array of NK cell activation receptors. Target cells may express ligands for a multitude of activation receptors, many of which signal through different pathways. How inhibitory receptors intersect different signaling cascades is not fully understood. This review focuses on advances in understanding how activation receptors cooperate to induce cytotoxicity in resting NK cells. The role of activating receptors in determining specificity and providing redundancy of target cell recognition is discussed. Using *Drosophila* insect cells as targets, we have recently examined the contribution of individual receptors. Interestingly, the strength of activation is not determined simply by additive effects of parallel activation pathways. Combinations of signals from different receptors can have different outcomes: synergy, no enhancement over individual signals, or additive effects. Cytotoxicity requires combined signals for granule polarization and degranulation. The integrin LFA-1 contributes a signal for polarization, but not degranulation. Conversely, CD16 alone or synergistic combinations, such as NKG2D and 2B4, signal for PLC-γ and PI3K–dependent degranulation.

Keywords

Innate Immunity; Natural killer cell; Signaling; Synergy

Introduction

NK cells are bone marrow–derived lymphocytes. Distinct from T and B cells, NK cells are controlled by a limited repertoire of germline–encoded receptors that do not undergo somatic recombination (1). Thus, NK cells represent an arm of the innate immune system. They participate in early defense against intracellular microbial infections and several types of tumors.

NK cells share a common killing mechanism with $CD8^+$ cytotoxic T lymphocytes (CTL), relying on directed exocytosis of secretory lysosomes that contain lytic proteins such as perforin, granzymes, and Fas ligand (2, 3). In addition, NK cells are a major source of cytokines such as tumor necrosis factor (TNF)-α and interferon (IFN)-γ. TNF-α initiates

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pro-inflammatory cytokine cascades (4), while IFN-γ promotes Th1 differentiation (5), enhances major histocompatibility class (MHC) I expression (6), and has potent anti-mycobacterial, anti-viral, and growth inhibitory effects (7, 8).

Innate resistance to intracellular pathogens mediated by NK cells involves both IFN-γ secretion and perforin–dependent target cell elimination. Animal models have provided compelling evidence for a direct role of NK cells in tumor surveillance, together with numerous reports implicating perforin and IFN-γ in such processes (9-11). NK cells may instruct and shape adaptive immune responses through cytokine release (12, 13) or by direct interaction with dendritic cells (14, 15). In addition to antibody–independent natural cytotoxicity, expression of CD16 (FcγRIIIA, the low affinity receptor for IgG) on a majority of blood NK cells renders them strong mediators of antibody-dependent cellular cytotoxicity (ADCC) against IgG–coated cells (16). Furthermore, NK cells can kill allogeneic cells in hematopoietic transplantation (17) and have clinical potential by conveying graft-versus-leukemia activity (18-20).

The prevailing view of NK cell activation is that they distinguish normal, healthy cells from sensitive target cells by a balance between signals from activating and inhibitory receptors (21-23). The net income of key positive and negative signaling events is thought to determine the capacity of NK cells to kill target cells. However, the precise molecular check-points where inhibitory signals abrogateactivating pathways are not well defined.

Taking a reductionistic approach to the study of activation in freshly isolated human NK cells, not activated by cytokines (hereafter referred to as resting NK cells), our lab has attempted to delineate the contribution of individual receptors to NK cell activation. Results have revealed that discrete activation steps are regulated by distinct receptors, and that a chain of events leading to NK cell effector functions can be triggered by a combination of co–activating receptors. This review covers new insights into the mechanism of NK cell activation and discusses how human resting NK cells recognize and eliminate target cells based on a multifaceted interplay between activating receptors. This offers a basis for understanding the contribution of activating receptors to specificity and redundancy in NK cell recognition.

Receptors on resting NK cells

Inhibitory receptors

In combat with rapidly evolving pathogens, NK cells must achieve specific recognition of infected or transformed cells, yet maintain tolerance for self. The ‘missing-self’ hypothesis (24) advocates a role for NK cell inhibitory receptors and target cell MHC class I expression in determining NK cell specificity. Inhibitory receptors expressed on human resting NK cells and their ligands are listed in Fig. 1.

Surprisingly, inhibitory receptors for classical MHC class I molecules in humans (Killer cell Immunoglobulin-like Receptors, KIR) and rodents (lectin-like Ly49) are structurally and evolutionary distinct. However, the genetic loci that encode receptors for MHC class I represent a striking example of convergent evolution (25). First, both KIR and Ly49 loci contain rapidly evolving genes that have arisen through extensive gene duplications. Second, the loci are highly polymorphic among different individuals at the level of gene content. Some alleles even encode an activating counterpart to an inhibitory receptor. Third, different receptors for MHC class I are expressed on distinct peripheral blood NK cell subsets. Other inhibitory NK cell receptors for human leukocyte antigens (HLA, or human MHC class I), such as NKG2A (CD159a) and LIR-1 (ILT2, CD85j), also display variegated expression patterns. Fourth, in spite of diversity in the extracellular ligand binding domains, NK cell

inhibitory receptors appear to use a common mechanism for inhibition. Upon engagement of classical MHC class I molecules (HLA-A, -B, -C), KIR can mediate inhibition of NK cell responses through recruitment of the phosphatase SHP-1 to phosphorylated, cytoplasmic immunoreceptor tyrosine-based inhibition motifs (ITIMs) (26, 27). Similarly, NKG2A, LIR-1, and mouse Ly49 receptors also contain cytoplasmic ITIMs that are capable of recruiting SHP-1 (28). The ligand of NKG2A is the non-classical MHC class I molecule HLA-E, which in turn serves as a gauge of classical MHC class I expression through its unique requirement for stabilization by leader peptides from HLA molecules (29-31). LIR-1 binds several alleles of classical MHC class I, in addition to the non-classical MHC class I molecule HLA-G (32, 33).

Inhibitory receptors for MHC class I are thought to mediate NK cell self-tolerance (34, 35). However, in spite of defective MHC class I expression, NK cells are self-tolerant in β2m–deficient mice (36, 37) or TAP–deficient humans and mice (38, 39). Remarkably, defective MHC class I expression leads to attenuated NK cell responses (36-39). Furthermore, a subpopulation of NK cells that lack known inhibitory receptors for self-MHC class I exists in normal mice, but display reduced responsiveness relative to NK cells expressing inhibitory receptors (40). Likewise, expression of inhibitory receptors specific for self-MHC confers greater responsiveness to NK cells, a property termed “licensing”, which requires functional ITIMs (41). Thus, NK cell reactivity is somehow “calibrated” by the MHC class I environment. The potency with which NK cells reject cells with aberrant MHC class I expression appears to correlate with the number and strength of inhibitory receptor – MHC class I interactions (41, 42).

Furthermore, non-MHC class I ligands for other ITIM-containing inhibitory receptors have been identified. The inhibitory lectin-like receptor KLRG1, expressed on a subset of NK cells (43, 44), binds members of the ubiquitously expressed cadherin family of cell-junction proteins in both humans and mice (45, 46). Loss of E-cadherin expression during metastasis and invasiveness of epithelial tumors has been suggested to facilitate NK cell surveillance of epithelial tumors (46). Another inhibitory lectin-like receptor, NKR-P1 (CD161), binds the related, but more widely expressed lectin–like molecule LLT1 in humans or other LLT1–homologues in mice (47-50). Subsets of human NK cells also express inhibitory, sialic acid–binding Siglec-7 (CD238) and Siglec-9 (CD239) receptors (51-53). Furthermore, the inhibitory receptor IRp60 (CD300a) is expressed by all resting NK cells (54), but ligands have not been identified. Whether inhibitory receptors for non-MHC class I ligands contribute to NK cell calibration has yet to be investigated.

Activating receptors

The discovery of ITIM-containing inhibitory receptors suggested that their interaction with MHC class I governed the specificity of NK cells for target cells. However, it has become clear that activation receptors contribute substantially to NK cell specificity. NK cells kill preferentially hematopoietic cells, whereas many tumors derived from other tissues are resistant to NK cells (55). This property has been exploited to improve the outcome of bone marrow transplantation. NK cells in T cell–depleted allogeneic hematopoietic grafts can mediate beneficial graft-versus-leukemia effects, but not graft-versus-host disease (18). These and other data imply that NK cell reactivity can be limited even in the absence of MHC class I on target cells. Although inhibitory receptors for non-MHC class I ligands may also control NK cells, the available evidence suggests that NK cells are not pre-wired to kill any encountered cell but depend on expression of sufficient ligands for positive recognition.

A large number of structurally distinct activating NK cell receptors have been characterized (1, 56). In contrast to inhibitiory receptors, most activating receptors are expressed by all NK cells. Furthermore, activating receptors induce diverse signaling cascades, whereas

inhibitory receptors appear to use a common mechanism for inhibition.. Some of the activating receptors expressed on human resting NK cells are listed, together with their ligands, in Fig. 2.

Activating receptors associated with immunoreceptor tyrosine-based activation motif (ITAM)-containing adaptor proteins propagate strong activation signals through recruitment of tyrosine kinases Syk and ZAP-70 (1, 56). Such receptors can be further subdivided into two groups; the first includes rapidly evolving receptors expressed on subsets of NK cells, such as KIR2DS, KIR3DS, and NKG2C (CD159c). The extracellular domains of these receptors are closely related to MHC class I–specific inhibitory receptor counterparts. These receptors associate with the ITAM-containing adaptor chain DAP12. Some activating KIRs bind classical MHC class I (57), whereas NKG2C binds HLA-E (29, 58). Generally, binding of activating receptors to MHC class I exhibits lower affinity than that of their related inhibitory receptor counterparts. Conservation of homologous activating and inhibitory receptor pairs through evolution may be important for maintaining immune system equilibrium (28), or may result from selective pressure imposed by pathogens (59, 60).

The second group of ITAM-associated receptors, including CD16, NKp30 (CD337), and NKp46 (CD335), are expressed on most resting NK cells. CD16 signals through the FcεRI γ-chain and the CD3 ζ-chain. CD16 binds the lower hinge region of IgG (61). Natural cytotoxicity receptors (NCRs) NKp30, NKp44, and NKp46 were identified for their role in natural cytotoxicity towards tumor cells (62). NKp44 is expressed only on IL-2–activated NK cells. NKp30 and NKp46 are not structurally related, but contain a transmembrane arginine residue, which forms salt-bridges with transmembrane aspartate residues in CD3 ζ-chain homodimers (63). Ligands for NCRs are not well defined. Experiments indicate that NKp46 binds viral hemagglutinins via sialic acid modifications of NKp46 (64, 65).

The perception that ITAM–mediated signaling induces potent NK cell activation, similar to how T cell and B cell activation depends on antigen receptor signaling, has been challenged. Although engagement of ITAM–containg receptors by specific mAbs induces lysis of FcR^+ cells in redirected lysis assays with IL-2–activated NK cells, this is not necessarily the case in assays with resting NK cells. Comparison of cytotoxicity by IL-2–activated and resting NK cells in redirected lysis assays revealed that mAbs to NKp30 and NKp46 do not efficiently trigger cytotoxicity by resting NK cells (66 and unpublished data). This is not due to an intrinsic defect in resting NK cell cytotoxicity, as mAbs to CD16 efficiently trigger lysis by resting NK cells (66). Under some circumstances, signaling by ITAM has even been shown to inhibit the function of other cell types (67, 68). Furthermore, signaling by ITAM is not a required component of NK cell effector function. Cytotoxicity towards certain target cells proceeds independently of ITAM, as NK cells from mice deficient in both Syk and ZAP-70 or a combination of the ITAM–containing adaptors DAP12, CD3 ζ-chain, and FcεR γ-chain can mediate cytotoxicity (69, 70).

A second category of activating receptors do not contain ITAMs or associate with ITAM–carrying adaptors. They include NKG2D (CD314), the CD2 family members CD2, 2B4 (CD244), CRACC (CD319), and NTB-A, and DNAM-1 (CD226). Human NKG2D associates with the adaptor protein DAP10 (ref. 1), which carries a phosphatidylinositol-3 kinase (PI3K) binding motif. The phosphorylated form of this tyrosine motif can bind the p85 subunit of PI3K and Grb2 (71). Ligands for NKG2D, such as MICA, MICB, and ULBP, are expressed on some tumor cells, and on infected or stressed cells (72). NKG2D ligands can be induced by genotoxic stress and stalled DNA replication, conditions that activate DNA damage checkpoint pathways (73).

CD2 signaling in NK cells is largely unknown. CD2 binds to LFA-3 (CD58). 2B4 (CD244) can recruit SAP and Fyn through cytoplasmic immunotyrosine–based switch motifs (ITSMs) (74, 75). The ligand of 2B4 is CD48, which is expressed on hematopoietic cells (76). CRACC and NTB-A also contain ITSMs, and are involved in homotypic interactions between hematopoietic cells (77-79). DNAM-1 is associated with leukocyte functional antigen (LFA)-1 in NK cells (80), is phosphorylated by a protein kinase C (PKC) (81), and binds to PVR (CD155) and Nectin-2 (CD112) (82). Similar to ITAM–associated receptors, receptors within this category are capable of inducing target cell lysis by IL-2–activated NK cells in redirected lysis assays (78, 79, 81, 83-87). Interestingly, analysis of mAbs to NKG2D, DNAM-1, 2B4 and CD2 with resting NK cells demonstrated that these receptors do not induce efficient lysis (66). Instead, these receptors may act in concert to induce NK cell activation (66).

Additional activating receptors expressed by all resting NK cells include CD7, CD44, CD59, KIR2DL4 (CD158d), and BY55 (CD160). CD7 encodes a cytoplasmic PI3K binding motif, binds SECTM1 or Galectin-1 (88, 89), can enhance NK cell cytokine secretion and β1-integrin–dependent adhesion to fibronection, but does not induce cytotoxicity (90). CD44 binds hyaluronan, a constituent of the extracellular matrix. Engagement of CD44 on does not induce cytotoxicity, but can co-stimulate CD16–dependent cytotoxicity by resting NK cells (91, 92). Engagement of CD44 on IL-2 or IL-12–activated NK cells can induce cytotoxicity (92). CD59 lacks a cytoplasmic tail but associates with NKp30 and NKp46 (93). Engagement of CD59 induces CD3 ζ-chain phosphorylation (93). Generally, CD59 binds complement C8 and C9, whereby formation of a membrane attack complex is prevented. Engagement of CD59 co-stimulates human NK cells (93). KIR2DL4 contains both a cytoplasmic ITIM and encodes a transmembrane arginine residue, through which it can associate with the FcεR γ-chain (94, 95). In contrast to other NK cell receptors, KIR2DL4 resides predominantely in intracellular vesicles (96). KIR2DL4 does not induce cytotoxicity but cytokine production by resting NK cells (97). Binding and internalization of soluble HLA-G, a ligand for KIR2DL4, induces cytokine secretion by resting NK cells in an ITAM-independent manner (96). Signaling by BY55 (CD160) is not well characterized. BY55 binds HLA-C and induces cytokine production by NK cells (98).

Integrins represent a different category of NK cell activating receptors, which are heterodimers of α and β subunits, such as αL and β2 subunits of LFA-1 (CD11a/CD18). LFA-1 binds intercellular adhesion molecules (ICAM)-1 through ICAM-5 (99). LFA-1 facilitates natural cytotoxicity and ADCC, as anti–LFA-1 blocking antibodies impair these processes (100-103). NK cells also express lower levels of β2-integrins Mac-1 (CD11b/CD18) and CD11c/CD18.

The β1-integrins expressed on NK cells, namely $\alpha_4\beta_1$ (very late antigen (VLA)-4, CD49d/CD29) and $\alpha_5\beta_1$ (CD49e/CD29), contribute activation signals upon binding to their ligands, vascular cell adhesion molecule (VCAM)-1 and fibronectin (104). Fibronectin coated on plates is sufficient to induce activation of mitogen–activated protein kinases (MAPK) in NK cells, specifically Erk and p38 (105). Interestingly, β_1 integrin engagement induces IL-8 production by NK cells, through a signaling pathway that involves Vav1/Rac1 and p38 MAPK activation (105).

Engagement of $\alpha_4\beta_1$ integrin activates Pyk2 and tyrosine phosphorylation of paxillin (106), and co-stimulates NK cell cytotoxicity (107). The complexity of intersecting signaling pathways in NK cells is illustrated by the inhibition of CD16–induced phospholipase D activation and degranulation, upstream of Ca^{2+} release, by co-ligation of $\alpha_4\beta_1$ integrin (108). The reason for this β1 integrin–mediated negative regulation is unknown. In addition, LFA-1–dependent migration of T cells is transactivated by $\alpha_4\beta_1$ through binding of paxillin

to the α_4 cytoplasmic tail and activation of Pyk2 (109). These data suggest that β_1 integrins may also regulate LFA-1–dependent signals in NK cells. Trans-regulation is mutual, as LFA-1 engagement upregulates ligand binding by β1 integrin (110).

Inhibitory NK cell receptors, which display variegated expression patterns on resting NK cell populations, may on the one hand potentiate NK cell effector function through calibration (34, 35) and on the other restrict activation towards targets expressing ligands for inhibitory receptors. Further, it is likely that cells in many tissues normally are not susceptible to NK cell mediated surveillance, because they do not express sufficient ligands to induce NK cell activation. Which of the many receptor–ligand interactions are sufficient or required for NK cell activation, and how receptors integrate to mediate NK cell activation requires further knowledge of the activation process.

To this end, we have developed a reductionist approach based on the use of *Drosophila* insect cells as target cells to study activation of normal, unmanipulated NK cells by physiological ligands in the absence of inhibitory receptor–ligand interactions (111). Unlike mammalian cells, insect cells are not expected to express many ligands for adhesion and activation of human NK cells. Therefore, they are better suited for investigations of the individual contribution of, and cross-talk among, NK cell receptors. Moreover, different assays were used to measure discrete steps in NK cell activation. With an emphasis on freshly isolated, resting NK cells, the goal of this approach is to characterize pathways leading to NK cell activation and define the minimal requirements.

Discrete steps in NK cell activation

Contact

Upon encounter with a sensitive target cell, several distinct molecular events lead to adhesion, granule polarization, degranulation, and cytokine production by NK cells (23). It is not clear which receptors provide initial signals upon NK cell contact with target cells. With insect cells, expression of ligands for CD16, 2B4, or LFA-1 alone was sufficient to induce signaling in resting NK cells, as assessed by receptor down-modulation and conjugate formation (111, 112).

Studies of T and B cells have suggested a prominent role for antigen–specific receptor signaling in initiating adhesion. Which of the many NK cell activation receptors signal upstream of LFA-1–mediated adhesion? We have observed that expression of human ICAM-1 on insect cells is sufficient to induce signaling-dependent adhesion by resting NK cells (111). Moreover, recombinant, plate-coated ICAM-1 also induces adhesion of resting NK cells (111). Together, this suggests that LFA-1 can provide autonomous signals for adhesion in resting NK cells.

Adhesion

Adhesion is thought to be a prerequisite for NK cell effector functions, providing stable contact with the target cell and leading to the formation of an immune synapse. Interaction of integrins with ligands on target cells must be regulated dynamically, as release from adherence is required for lymphocute movement.

In the insect cell system, adhesion was evaluated by formation of conjugates between NK cells and target cells expressing specific ligands. Engagement of LFA-1 by ICAM-1 or ICAM-2 is sufficient to induce adhesion by human resting NK cells (111, 112). LFA-1–dependent adhesion can be augmented by stimulation with exogenous IL-2 and IL-15 (111). Resting NK cell adhesion is also augmented by the co-expression of ligands for CD2, CD16, and 2B4 (111, 112). Engagement of CD2, CD16, or 2B4 alone does not induce adhesion

(111, 112). Use of pharmacological inhibitors of the actin cytoskeleton, Src-family kinases, or PI3K indicated a signaling–dependent role of CD2 and 2B4 in enhancing LFA-1–dependent adhesion (111). In LFA-1–deficient mice, IL-2–activated NK cells have a profound deficiency in target cell adhesion (113). Interestingly, the immunoglobulin superfamily molecule CD44 facilitates LFA-1–dependent adhesion, as LFA-1–dependent adhesion is diminished in CD44–deficient mice (113).

Adhesion and signaling by LFA-1 is a carefully orchestrated process. Activating receptors may provide inside-out signals, which increase LFA-1 affinity through conformational changes (Fig. 3). Alternatively, signals from activating receptors may also induce LFA-1 clustering, whereby LFA-1 avidity is enhanced (Fig. 3). In the resting state, the αL and β2 cytoplasmic domains are close to each other and the extracellular domain closed, but either inside-out signaling or ligand binding induces an extended conformation of the extracellular domain coupled to a spatial separation of the cytoplasmic domains (114). In the ligand binding conformation, LFA-1 itself can transduce outside-in signals (Fig. 3). Apart from LFA-1 outside-in signals themselves promoting LFA-1 adhesion (thereby conferring inside-out signals) (111, 112), evidence of inside-out signaling by other NK cell receptors has not been directly assessed in terms of LFA-1 affinity or avidity. Data remain circumstantial, demonstrating a combined contribution by LFA-1 and other receptors such as CD2, CD16, and 2B4 in augmenting LFA-1–dependent adhesion.

Many signaling molecules and pathways have been implicated in modulation of LFA-1 affinity (115). In other cell types, LFA-1 affinity is intimately coupled to regulation of the actin cytoskeleton. For example, LFA-1 affinity can be promoted by calpain, a Ca^{2+}–dependent protease (116). Calpain–mediated cleavage of talin, a cytoskeletal component, produces a talin fragment that binds the cytoplasmic tail of integrin β chains, thereby inducing separation of the cytoplasmic tails and augmenting LFA-1 affinity (114, 117). Evidence suggests competition for β chain binding between talin and another actin-binding protein, filamin. Binding of filamin inhibits integrin affinity, talin and filamin binding sites on the β chain overlap, and talin binding might be promoted by phosphorylation of threonine residues in the filamin binding site that would displace filamin (118, 119).

In T cells, a distinct role has been described for the GTPase Rap1 in regulation of LFA-1 avidity. In resting T cells, a fraction of LFA-1 is phosphorylated on αL Ser1140. Phosphorylation of this residue is required for induction of LFA-1 clustering by Rap1 (120). Activation of Rap1 by chemokine stimulation or T cell receptor engagement can induce RAPL binding to the αL cytoplasmic tail, which in turn leads to redistribution of LFA-1 to the immunological synpase (121).

Although genetic evidence suggest a major contribution of LFA-1 to NK cell adhesion, the potential contribution of other receptors to NK cell adhesion remains to be assessed.

Polarization

For cytotoxic cells in general, polarization of the secretory lysosomes (also called cytotoxic granules) precedes target cell cytotoxicity (3). Early studies of the interaction between NK cells and sensitive target cells revealed that adhesion was accompanied by NK cell polarization of the actin cytoskeleton, Golgi apparatus, and microtubules towards the target cell interface (122-124). Live cell imaging experiments during natural cytotoxicity by lymphokine–activated killer cells demonstrated that NK cells establish cytoskeletal polarity in a stepwise fashion, suggesting a series of checkpoints, as opposed to cytolytic T cells where antigen induces rapid and robust cellular polarity (125).

In T cells, engagement of the antigen receptor is required to induce polarization (126). The receptor–ligand interactions required or sufficient for polarization in NK cells were until recently undefined. To assess polarization, we visualized perforin in NK cells that had formed conjugates with insect cells. Insect cells expressing CD48, the ligand of 2B4, or coated with rabbit IgG (to engage CD16) did not induce polarization in resting NK cells (112). However, engagement of LFA-1 by ICAM-1 on insect cells is sufficient to induce polarization of perforin–positive granules towards the interface of insect cells and NK cells, either resting or IL-2–activated (112, 127). Beads coated with ICAM-1–Fc fusion proteins also induced granule polarization by resting NK cells, implying that signaling by LFA-1 alone is sufficient (112). The data imply a central role for LFA-1, not only in target cell adhesion but also in signaling for cytotoxicity, and suggest that LFA-1 can prime NK cells for cytotoxicity (Fig. 4A). However, granule polarization can also occur in the absence of LFA-1 engagement, as a combination of signals from CD16 and 2B4 induced polarization (Fig. 4B).

The signals that regulate granule polarization in NK cells are not well defined, but expression of dominant–negative Rac1 and RhoA does inhibit polarization of perforin in IL-2–activated NK cells, whereas overexpression of Vav1 enhances polarization (128). Further, pharmacological inhibitors of PI3K inhibited polarization of perforin and suppressed cytotoxicity in an IL-2–dependent NK cell line (129). As highlighted, LFA-1 is sufficient for both adhesion and polarization in NK cells. In both IL-2–activated NK cells and T cells, engagement of LFA-1 by ICAM-1 induces activation of a Vav–Rac–PAK1 pathway (130-132). Furthermore, chemoattractans can induce PI3K activity associated with LFA-1 in a manner dependent on association of the Src-family kinase Fyn with the LFA-1 cytoplasmic tail (133). In T cells, Fyn has been demonstrated to be upstream of Vav1–mediated signals for T cell polarization (134). Supporting these findings, Fyn-deficient mice have defective tubulin cytoskeleton rearrangements in T cells and granule polarization in mast cells (135, 136). NK cell function has been studied in Fyn–deficient mice. Notably, Fyn is required for efficient, NK cell-mediated lysis of target cells which lack both self-MHC class I molecules and ligands for NKG2D (137). In contrast, NK cell inhibition by the MHC class I-specific receptor Ly49A was independent of Fyn, suggesting that Fyn is specifically required for NK cell activation (137). Future studies using genetic approaches will hopefully elucidate the signaling pathways responsible for granule polarization in NK cells.

Degranulation

Perforin and other cytotoxic proteins are constitutively expressed by resting NK cells and stored in specialized secretory lysosomes (112, 138). Degranulation (exocytosis of secretory lysosomes) can be measured by the release of hexosaminidase or granzyme B into supernatants. Another assay, which can quantitate degranulation at the single cell level, is based on appearance of CD107a (LAMP-1) at the cell surface upon degranulation. CD107a is a lysosomal membrane protein that colocalizes with perforin in secretory lysosomes and redistributes to the cell surface when granules fuse with the plasma membrane (112, 139). Resting NK cell degranulation, as assessed by CD107a surface staining, is observed within minutes of mixing with sensitive target cells (112), and parallels the rapid mobilization of calcium by receptor engagement (66) or interaction with target cells (125).

So far, we have ascribed a prominent role to LFA-1 in the early steps of NK cell activation, including contact, adhesion and polarization. However, we have found that engagement of LFA-1 by ICAM-1 or ICAM-2 on insect cells is neither sufficient nor required for degranulation (112). Instead, engagement of CD16 by antibody-coated insect cells is sufficient for degranulation in the absence of LFA-1–dependent signaling (112). In another report, perforin release induced by soluble ICAM-2 and ICAM-3 Fc fusion proteins, but not

ICAM-1–Fc, was observed in a $CD8^+$ subset of human NK cells (140). Because the Fc portion of the ICAM fusion proteins was derived from human IgG1, it is not possible to exclude co-engagement of and activation by CD16, as has been described with other human IgG1 fusion proteins (141).

In contrast to the degranulation induced by CD16 ligation, ligation of NKG2D or 2B4 by Abs bound to the FcR^+ mouse cell line P815 did not induce degranulation (66). When mAbs were combined, NKG2D and 2B4 synergistically induced degranulation in resting NK cells (66). However, as P815 cells express mouse ICAM-1, which binds human LFA-1 (142), it is possible that recognition of mouse ligands by human NK cells contributes to activation.

NK cell degranulation requires calcium and is induced by PKC and G protein–dependent pathways (143). Patch clamp experiments have shown that cytosolic Ca^{2+} is sufficient to induce degranulation in an NK cell line (144). PLC-γ2–deficient mice have defective NK cell natural cytotoxicity and ADCC, and display increased viral loads upon infection with cytomegalovirus (145-147). Although NK cells from PLC-γ2–deficient mice polarize granules towards sensitive target cells, no intracellular calcium mobilization is observed after engagement of multiple activating receptors, and degranulation induced by sensitive target cells is abolished (147). While mouse NK cells predominantely express PLC-γ2, human NK cells express both PLC-γ1 and PLC-γ2 (146, 148). Experiments in our laboratory also suggest that inhibition of PLC-γ with the pharmacological compound U73122 abrogates resting NK cell degranulation induced by both ITAM–dependent and –independent pathways (Fig. 5). Inhibition of Src-family kinases by PP2 also blocks degranulation. (Fig. 5). Further, pharmacological inhibition of PLC-γ by U73122 also inhibits Ca^{2+} mobilization and cytotoxicity induced by mAb–mediated crosslinking of CD16 alone, or NKG2D and 2B4 together, in resting NK cells (unpublished data).

Future studies should elucidate how PLC-γ–dependent Ca^{2+} mobilization and PKC activation in turn leads to degranulation. In NK cells, the proteins responsible for these downstream processes are largely unknown.

NK cell receptor cooperation for cytotoxicity

So far, data obtained with the insect target cell system suggest that no single receptor–ligand interaction is sufficient to trigger all activation steps. Conceptually, one could envisage that certain steps might be required for triggering of consecutive events. For example, a sequence of adhesion followed by granule polarization and degranulation. However, *in vitro* mixing experiments with insect cells and resting NK cells suggest that receptors may trigger discrete activation steps independently of each other. Thus, NK cell activation does not necessarily follow a sequence of events, but is guided by the receptor or receptor combinations engaged upon encounter with target cells. Admittedly, requirements for NK cell activation could be more stringent *in vivo*, under conditions of shear flow and limited ligand availability.

Studies with insect cells expressing ligands of human NK cell receptors have shown that individual receptor–ligand interactions are not sufficient to induce efficient cytotoxicity by resting NK cells. Although degranulation appears to be necessary for cytotoxicity by resting NK cells, it is not synonymous with target cell lysis.e Neither granule polarization nor degranulation alone is sufficient for cytotoxicity. Rather, combinations of NK cell receptors cooperate to induce efficient elimination of target cells.

ADCC

Antibody–coated target cells trigger strong CD16–mediated NK cell activation. In addition to macrophages and some T cells with a memory phenotype, blood and spleen $CD56^{dim}$ NK

cells express CD16 (149). In the blood, $CD56^{dim}$ NK cells constitute more than 90% of the total NK cell population. CD16 expression is virtually absent from lymph node and tonsil NK cells, where $CD56^{bright}$ NK cells are enriched (149, 150).

Previous studies using cells from LFA-1–deficient patients or experiments with LFA-1–blocking mAbs demonstrated a role for LFA-1 in ADCC. Individuals with leukocyte adhesion disorder (LAD), a syndrome due to β2-integrin subunit deficiency, suffer severe recurrent bacterial infections, susceptibility to herpes simplex virus (HSV) infections, and impaired immunity (151-153). Severely affected patients often die of infection in childhood or early adulthood unless bone marrow transplantation is successfully accomplished. NK cells from LAD patients display attenuated ADCC (151, 154) and anti-LFA-1 blocking antibodies inhibit lysis of anti-CD16 expressing hybridomas (102, 103). The interpretation of these results used to be that LFA-1 is required to provide target cell adhesion, in order for activating receptors such as CD16 to trigger NK cell cytotoxicity. In contrast, we have described that antibody-coated insect cells are sufficient to induce CD16–mediated degranulation by resting human NK cells, as determined by granzyme B release and induction of CD107a surface expression (112 and Fig. 6). CD16–mediated degranulation occurs in spite of very low target cell adhesion. Furthermore, examination of conjugates between resting NK cells and IgG–coated target cells did not reveal granule polarization. Alone, engagement of LFA-1 by ICAM-1 on insect cells is sufficient to induce low adhesion and granule polarization (Fig. 4). Efficient ADCC by resting NK cells requires the combined presence of IgG and expression of human ICAM-1 on insect cells (112 and Fig. 6). The results define two separable signals for adhesion/polarization (LFA-1) and degranulation (CD16) that are required for target cell killing (Fig. 6). Notably, ICAM-1 expression on target cells does not increase CD16–induced degranulation. Results are also in agreement with a study of T cells (155), which concluded that LFA-1–ICAM-1 interactions are dispensable for degranulation, but essential for effective target cell lysis through enhancement of TCR–dependent granule polarization towards target cells.

In the absence of LFA-1 engagement, CD48 expression by insect cells enhances ADCC (112 and Fig. 6). At comparable IgG concentrations, co-engagement by target cell ligands of CD16 and 2B4 induced target cell killing as efficiently as co-engagement of CD16 and LFA-1. The mechanisms used by 2B4 or LFA-1–dependent co-stimulation of ADCC are different. In contrast to LFA-1–mediated co-stimulation, IgG in combination with CD48 expression on insect cells does not induce strong adhesion. Instead, co-engagement of CD16 by IgG and 2B4 by CD48 augments signals for polarization (Fig. 4B) and degranulation (Fig. 7). CD48–mediated co-stimulation lowered the IgG concentration required to induce resting NK cell degranulation. In terms of early signaling events, co-crosslinking of CD16 and 2B4 by specific mAbs synergistically augments intracellular calcium mobilization relative to cross-linking of CD16 alone (66). MAb–mediated co-engagement of other NK cell receptors, such as NKG2D, DNAM-1, and CD2 also augmented CD16–induced calcium fluxes (66). Therefore, expression of ligands for other activating NK cell receptors might also synergistically co-stimulate CD16–triggered degranulation and reduce the concentration of IgG required to trigger resting NK cell degranulation (Fig. 7). Of interest, ITAM–mediated signals from different receptors do not enhance each other, as co-engagement of NKp46 with CD16 does not result in enhanced responses (66).

Natural cytotoxicity

A large number of receptors have been implicated in natural cytotoxicity (i.e. antibody–independent NK cell cytotoxicity). Two commonly used approaches to characterize NK cell activating receptors have been to identify mAbs that interfere with NK cell–mediated lysis of sensitive target cells and evaluate whether mAbs to such NK cell structures trigger redirected lysis of the FcR^{+} target cell line P815. In such experiments, IL-2–activated NK

cells are commonly used as effectors. Although mAbs to CD16 induce lysis of P815 cells by both resting and IL-2–activated NK cells, mAbs to other NK cell receptors such as NKp46, NKG2D, 2B4, DNAM-1, and CD2 do not efficiently trigger lysis by resting NK cells (66). Nonetheless, K562 cells trigger both degranulation and lysis by resting NK cells, suggesting no impairment of natural cytotoxicity in resting NK cells *per se* (112).

Upon closer examination, we found that mAb–mediated crosslinking of NKp46, NKG2D, 2B4, DNAM-1, and CD2 only induces weak intracellular Ca^{2+} mobilization, as compared to Ca^{2+} mobilization induced by mAb–mediated crosslinking of CD16 (66). However, co-crosslinking of specific, pairwise combinations of receptors can induce synergistic Ca^{2+} mobilization (66). Results revealed a hierarchy of receptors for activation of resting NK cells, as depicted (Fig. 8A). The unique pattern of receptor combinations that provide synergy is consistent with the use of different signaling modules by each receptor to induce activation. We propose the term “co-activation” receptors, as they do not by themselves induce strong activation signals, but depend on co-engagement of other co-activating receptors for activation of NK cell function (66). Generally, the same combinations of mAbs that synergize for Ca^{2+} mobilization also enhance resting NK cell cytotoxicity and cytokine production (66). Moreover, while engagement of neither receptor alone induces degranuation, co-engagement of 2B4 with NKG2D or DNAM-1 by mAbs can induce strong synergistic signals that lead to degranulation (66, unpublished data, and Fig. 8B). Thus, we speculate that receptor co-activation as observed between 2B4 and NKG2D, or 2B4 and DNAM-1, may be responsible for the ITAM–independent NK cell cytotoxicity observed in mice deficient in both SYK and ZAP-70 (69). In these mice, NKG2D can contribute to target cell lysis by IL-2–activated NK cells (156).

Recently, it was reported that engagement of NKG2D with mAbs is not sufficient to induce activation of resting NK cells (157). However, binding to MICA and ULBP1 Fc fusion proteins induced activation of resting NK cells, as assessed by CD25 up-regulation and proliferation. Insect cells expressing ligands of NKG2D will be useful to define the basis for this discrepancy.

So how might NKG2D and 2B4 signals synergize for PLC-γ recruitment, Ca^{2+} mobilization, degranulation, and cytotoxicity? Through DAP10, NKG2D can recruit PI3K (158). In IL-2–activated NK cells recruitment of PI3K by DAP10 leads to activation of Vav, Rho family GTPases, and PLC-γ (159). In resting NK cells, this pathway only induces a minor, but reproducible Ca^{2+} mobilization that can be inhibited by wortmannin or Ly294002, which are pharmacological inhibitors of PI3K (Fig. 9A and unpublished data). Similar to NKG2D, 2B4 activates PLC-γ in IL-2–activated NK cells (160). Unlike NKG2D and CD16 crosslinking, however, Ca^{2+} mobilization induced by 2B4 crosslinking is insensitive to PI3K inhibitors in resting NK cells (Fig. 9A). Synergy of 2B4 and NKG2D–DAP10 signals could could be due to enhanced PI3K–mediated membrane recruitment of PLC-γ through the PLC-γ pleckstrin homology (PH) domain. Surprisingly, the synergistic Ca^{2+} mobilization induced by NKG2D and 2B4 co-activation is insensitive to PI3K inhibitors in resting NK cells (Fig. 9A). Therefore, the NKG2D signal that augments Ca^{2+} mobilization in co-ordination with 2B4 signals is PI3K–independent. Further, although PI3K inhibition only partially inhibits CD16 and has no effect on NKG2D and 2B4 synergistic Ca^{2+} mobilization, it abolishes resting NK cell degranulation (Fig. 7B) and cytotoxicity (unpublished data). The data demonstrate that NKG2D and 2B4 co-activation of Ca^{2+} mobilization is PLC-γ–dependent and PI3K–independent, while resting NK cell cytotoxicity requires both PLC-γ and PI3K for degranulation. In line with these finding and providing mechanistic insights, a recent study (71) showed that NKG2D–DAP10 recruitment of both a Grb2–Vav complex and the p85 subunit of PI3K is required for NKG2D–mediated cytotoxicity in IL-2–activated NK cells. Substantiating these findings, PLC-γ is activated independently of PI3K, but associates with

Vav and SLP-76 in activated human mast cells (161). Thus, PLC-γ and PI3K are emerging as two critical signaling components for NK cell degranulation, where requirement for PI3K appears to be be downstream of PLC-γ activation.

It should be emphasized that the outcome of specific receptor engagement on NK cells is not clear-cut. NK cell responses are not merely a function of engaged receptors, but also represent the expression and distribution of intracellular signaling molecules present in any given NK cell. The availability of signaling components is influenced by cell maturation stage, and potentially modulated by inhibitory receptor calibration and inflammatory signals. These factors combine to fine-tune and provide distinctiveness to the reactivity of individual NK cells. 2B4 is one example. In mature human NK cells, 2B4 can induce SAP–dependent NK cell activation (74, 75, 162). In NK cells from X-linked lymphoproliferative disease (XLP) patients, which have a deficiency in SAP, 2B4 is inhibitory (162). Consequentially, 2B4–dependent killing of autologous EBV infected B cells is abolished in SAP–deficient XLP patients (162). Moreover, immature NK cells contain low levels of SAP and engagement of 2B4 can therefore mediate tolerance of immature NK cells that have not acquired expression of inhibitory receptors for MHC class I (163). These data to some extent corroborate divergent findings with 2B4–deficient and SAP–deficient mice, in which 2B4 has been attributed both positive and negative signaling functions (164, 165). Adding further complexity, a structural homologue of SAP that competes for binding to ITSMs has been described. EAT-2 is also expressed in human NK cells and can bind ITSMs of 2B4 and other CD2-related receptors (166). In an NK cell line, Tassi *et al*. (166) observed that EAT-2 preferentially binds 2B4 in non-activated cells, whereas SAP binds better after cell activation. Tentatively, 2B4–mediated activation could therefore be dynamically regulated by SAP expression and competition for ITSM binding with EAT-2.

In conclusion, resting NK cells are not inherently non-responsive, but the regulation of their activation is far more stringent than that of IL-2–activated NK cells. Receptors can signal independently in resting NK cells, but cytotoxicity requires a combination of signals for adhesion, granule polarization, and degranulation, supplied by two or more interactions between different receptor–ligandpairs. It appears that no receptor alone, but co-engagement of certain combinations of co-activating receptors induces efficient cytotoxicity.

Intersection of activation pathways and inhibitory signals

Considering the many different kinds of signals delivered by NK cell activation receptors, and the strong synergies among activation signals described here, it is remarkable that inhibitory receptor engagement by MHC class I on target cells can prevent cytotoxicity so efficiently. It is generally thought that phosphorylation of ITIMs in the cytoplasmic tail of inhibitory receptors is dependent on Src-family kinases that are participating in the activation cascade. Accordingly, co-clustering of activation receptors and inhibitory receptors may lead to trans-phosphorylation of ITIMs, recruitment of the tyrosine phosphatases SHP-1 and SHP-2, which then abort signaling cascades by dephosphorylating multiple tyrosine phosphorylated effector molecules. However, this view is not supported by any direct experimental evidence. Furthermore, it would put inhibitory receptors at a disadvantage by placing them downstream of initial activation signals.

One of the great advantages of the insect cell system is to be able to study NK cell activation in the absence of complicating inhibitory interactions. MHC class I–deficient mammalian cells do not offer this advantage, as inhibitory receptors for several non-MHC class I ligands may also contribute to negative regulation of NK cells (Fig. 1). In addition, the insect cell system is perfectly suited to study the contribution of individual inhibitory receptors.

Expression of MHC class I molecules and β_2-microglobulin in insect cells results in efficient transport of empty class I to the cell surface (167). This property offers the opportunity to load MHC class I molecule with defined peptides. Using this system, we have shown that human inhibitory KIR cluster at the interface of NK cells with insect cells expressing peptide-loaded HLA-C, even in the absence of ICAM-1–mediated adhesion (168). Furthermore, interaction of KIR with HLA-C on insect cells is functional, as shown by the inhibition of LFA-1–induced signals (127). These results show that signals delivered by LFA-1 are sensitive to inhibition by KIR. Together with other data, these findings are leading to a revised view of the ITIM–dependent inhibitory mechanism.

A "functional trapping" experiment with a substrate–trappping mutant of SHP-1 expressed in the NK cell line YTS led to the identification of Vav1 as a target of SHP-1–mediated dephosphorylation during inhibition by KIR–HLA-C interaction (169). Interestingly, Vav1 and its close relatives Vav2 and Vav3 have been implicated in different signaling pathways downstream of several NK cell activation receptors, such as CD16, NKG2D (170), 2B4 (74), and β_2 integrin (131). Therefore, it is possible that dephosphorylation of Vav during inhibition by KIR is a way to stop different signaling pathways at a common point. Trapping of Vav1 was insensitive to cytochalasin D, suggesting that dephosphorylation of substrates occurs independently of actin polymerization (169). As phosphorylation of activation receptor 2B4 is dependent on actin polymerization, the inhibition mediated by KIR may preceed full engagement of receptor 2B4. The revised view of the inhibitory pathway is one where KIR operates independently of activation signals, thereby preventing activation at a very early step, including signals delivered by LFA-1.

Much remains to be learned about the precise way in which ITIM–based inhibitory signals intersect the many signals received by NK cells, and how inhibitory receptors control the various steps in NK cell activation, such as inside-out signals to LFA-1, signals for granule polarization, and the many combinations of synergistic signals that induce cytokine release and cytotoxicty.

Relevance to *H. sapiens*

Moving away from reductionist experiments into the more complex *in vivo* environment, what can knowledge of the regulation and function of NK cells receptors tell us about the evolutionary forces shaping NK cell receptor repertoires? And how can we benefit from this knowledge to exploit NK cell effector functions in the clinic?

Host–pathogen interface

NK cells mediate anti-viral defense. Hence, viruses have evolved numerous strategies to avoid NK cell recognition of virally infected cells. The rapid evolution of MHC class I receptors provides insight into an interesting strategy in the conflict opposing microorganisms and immune system. Viral subversion of T cell–mediated immune surveillance through MHC class I down-regulation can render infected cells susceptible to NK cell lysis. Therefore, NK cell inhibitory receptors are targeted by viral immune evasion strategies. Viruses may express MHC class-like decoy molecules that can engage certain NK cell inhibitory receptors or enhance expression of endogenous MHC class I molecules. Human CMV encodes UL18 which can bind LIR-1 with high affinity (33, 171), and UL40 which can enhance expression of endogenous HLA-E (a ligand for NKG2A) (172). However, due to the variegated expression pattern of inhibitory receptors, effective NK cell inhibition requires co-ordinated targeting of several inhibitory modalities. Individuals display considerable variation in the numbers of inhibitory receptor–MHC class I interactions. Most individuals possess a minimum of three interactions (KIR2DL–HLA-C, NKG2A–HLA-E, LIR-1–HLA class I) that would have to be circumvented by pathogens for

evasion of NK cell activation. Thus, the variation in NK cell inhibitory receptor interactions provides robustness to the organisms both at the individual and species level. Conceivably, viral evasion of inhibitory receptors could be a driving force for the rapid genetic evolution of these receptor systems (173). Genetic associations between combinations of KIR and HLA genotypes and susceptibility to viruses imply that KIR–HLA interactions are crucial to anti-viral immunity (174).

As a central mediator of NK cell adhesion and granule polarization, LFA-1 should be an attractive target for viral escape of NK cell recognition. Indeed, Kaposi's sarcoma herpesvirus encodes a protein that selectively down-regulates ICAM-1 expression on virally infected cells (175). However, at least for ADCC, co-stimulation by other receptors can overcome requirements for LFA-1–dependent adhesion and signaling in NK cell cytotoxicity (112).

To avoid viral escape from NK cell–mediated immunosurveillance, redundancy in recognition systems employed by NK cells is essential. Viral proteins interfere with expression of ligands for NK cell receptors NKG2D and PVR. Human cytomegalovirus (CMV) encodes UL16 and UL141, proteins that interfere with expression of ULBPs and CD155, respectively, suggesting that NKG2D and DNAM-1 contribute to NK cell–mediated immune responses to CMV (176, 177). Notably, both NKG2D and DNAM-1 can bind several divergent ligands, complicating viral efforts to block recognition by ligand down-regulation. Using a different mechanism, human CMV also encodes pp65, a protein that can directly bind the activating receptor NKp30 and block NKp30–dependent immune activation (178). Our results suggest that human resting NK cells can be activated by several, non-overlapping combinations of activating receptors (66), signifying redundancy in NK cell recognition. Plausibly, redundancy could be a driving force in the evolution of NK cell activating receptor repertoires for natural cytotoxicity.

It is possible that ADCC my contribute to resistance to viruses, in addition to the protection provided by natural cytotoxicity. However, individuals with mutations leading to CD16–deficiency or polymorphisms with reduced binding of IgG are not reported to have increased viral infections, but increased incidence of autoimmunity (179, 180). CD16 mutations have been reported that do not abrogate NK cell–mediated ADCC, but impair natural cytotoxicity and viral immunity, suggesting a potential ADCC–independent role for CD16 (181). Nonetheless, other studies suggest that NK cell–mediated ADCC might contribute to HSV, human immunodeficiency virus (HIV)-1, and influenza virus protection (182-185). Physiologically, the availability of IgG against target cell epitopes is probably limiting in early adaptive immune responses. Under these circumstances, co-stimulation of CD16 signals by both receptors that provide strong adhesion/polarization (LFA-1) and receptors that augment signals for degranulation (2B4 and other co-stimulating receptors) are likely necessary for efficient ADCC.

Finally, there is ample evidence that NK cells contribute to protection from intracellular bacteria and parasites (186), which may also be a driving force in evolution of NK cell receptors.

Clinical perspectives

NK cells could potentially be exploited in cancer immunotherapy. Alloreactive NK cells have beneficial anti-tumor effects in hematopoietic stem cell transplantation of patients with acute myeloid leukaemia (18). In addition, NK cells from KIR–HLA incompatible donors can kill melanoma and renal cell carcinoma cells *in vitro* (187). Importantly, delineating the activation of NK cells may allow prediction of tumor cell sensitivity to NK cell killing based

on phenotypic analysis of ligand expression, and thus predict efficacy of NK cell–mediated immunotherapy.

Cytokines such as type I IFN, IL-2, IL-12, and IL-18 enhance NK cell activity (12, 188). Controlled exogenous administration could provide a valuable tool for up-regulating NK cell effector functions. Moreover, therapeutic blockade of inhibitory receptor–ligand interactions could potentially allow reactivity from wider NK cell subsets and facilitate the use of NK cells in an autologous setting.

In addition to inhibitory receptor blockade, natural cytotoxicity against tumors can potentially be aided by preferential engagement of activating NK cell receptors. One strategy is to design bispecific proteins that can simultaneously engage tumor markers and NK cell activating receptors. In a recent article a fusion protein of ULBP2 and an anti-CD138 antibody fragment was described that can mediate antitumor activity in a xenograft model of multiple myeloma (189). Another approach would be to adoptively transfer gene-modified NK cells with targeted chimeric receptors for tumor antigens fused to potent activating NK cell receptor components, such as a CD4-ζ chain chimera that binds to HIV-infected cells (190).

We would argue that engagement of CD16 would be the most applicable and affordable approach to harnessing NK cells for immunotherapy. Indeed, several human IgG mAb–based treatments are increasingly applied in immunotherapy against haematological and non-haematological malignancies. Examples include rituximab (anti-CD20) for B cell lymphomas, alemtuzumab (anti-CD52/CAMPATH) for B cell chronic lyphocytic leukemia, trastuzumab (anti-HER2) for breast cancer, and adecatumumab (anti-EpCAM) for prostate and breast cancer. The extent to which these antibody–based tumor therapies are NK cell–mediated is not clear, but all are capable of triggering NK cell–mediated cytotoxicity *in vitro* (191, 192). The most convincing evidence for CD16 and possible NK cell involvement comes from studies with rituximab. In patients undergoing treatment for large-cell non-Hodgkin's lymphoma, rituximab (anti-CD20) administration induces NK cell degranulation *in vivo* (192). A positive correlation between better clinical responses to rituximab and CD16 polymorphisms that result in higher affinity for IgG have been noted (193, 194). Moreover, in a study of a combination therapy of rituximab and recombinant IL-2, NK cell expansions correlated with a favourable clinical response (195). In primary breast cancer, a recent report advocates an *in vivo* role for NK cells in the mechanisms of trastuzumab action, since treatment significantly increased numbers of tumor-associated NK cells (196). A consequence of NK cell activation by ADCC is production of T cell recruiting chemokines, which can be measured in sera from patients with clinical benefit from rituximab treatment and have been shown to induce T cell migration (197). Thus, NK cell–mediated ADCC could promote beneficial adaptive immune responses.

Appreciation of NK cell biology could encourage new, combinatorial therapeutic approaches. Specifically, enhancing NK cell effector function through upregulation of inducible ligands, for receptors such as LFA-1 or NKG2D might be feasible. Proinflammatory cytokines can rapidly upregulate ICAM-1 on several tissues (198), while NKG2D ligands can be induced by radiation or chemotherapeutic drugs (199). In combination with specific engagement of effective triggering receptors on NK cells, desired cytolytic activity could be accomplished.

Concluding remarks

In conclusion, different receptors contribute discrete signals leading to cytotoxicity by resting human NK cells. An important goal is to distinguish the contribution of different components of NK cell effector function in immune responses. Many challenges remain.

Which receptor signals and activation steps are sensitive to inhibitory receptor signals? Further studies need to address the requirements for, and contribution by, individual NK cell receptors to cytokine release. With resting NK cells as a reference point, it will be interesting to determine how various exogenous cytokines modulate specific steps in NK cell activation pathways. Understanding the regulation of primary NK cells will hopefully lead to promising strategies for harnessing NK cell cytotoxicity in a clinical setting.

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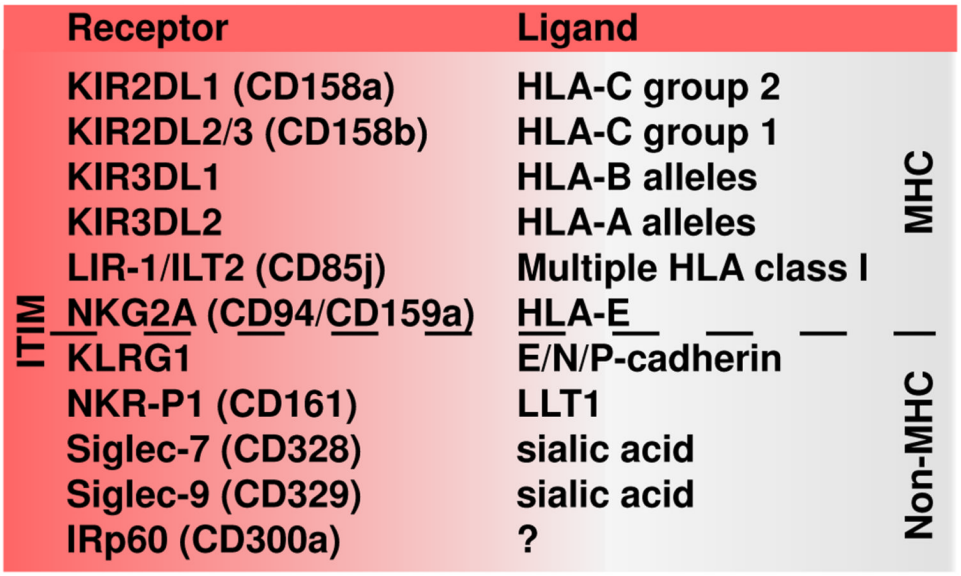

Fig. 1. Inhibitory receptors expressed on human peripheral blood NK cells

Inhibitory receptors expressed by freshly isolated, resting NK cells and their ligands are listed. KIR, NKG2A, LIR-1, KLRG1, NKR-P1, Siglec-7, and Siglec-9 are only expressed by subsets of NK cells.

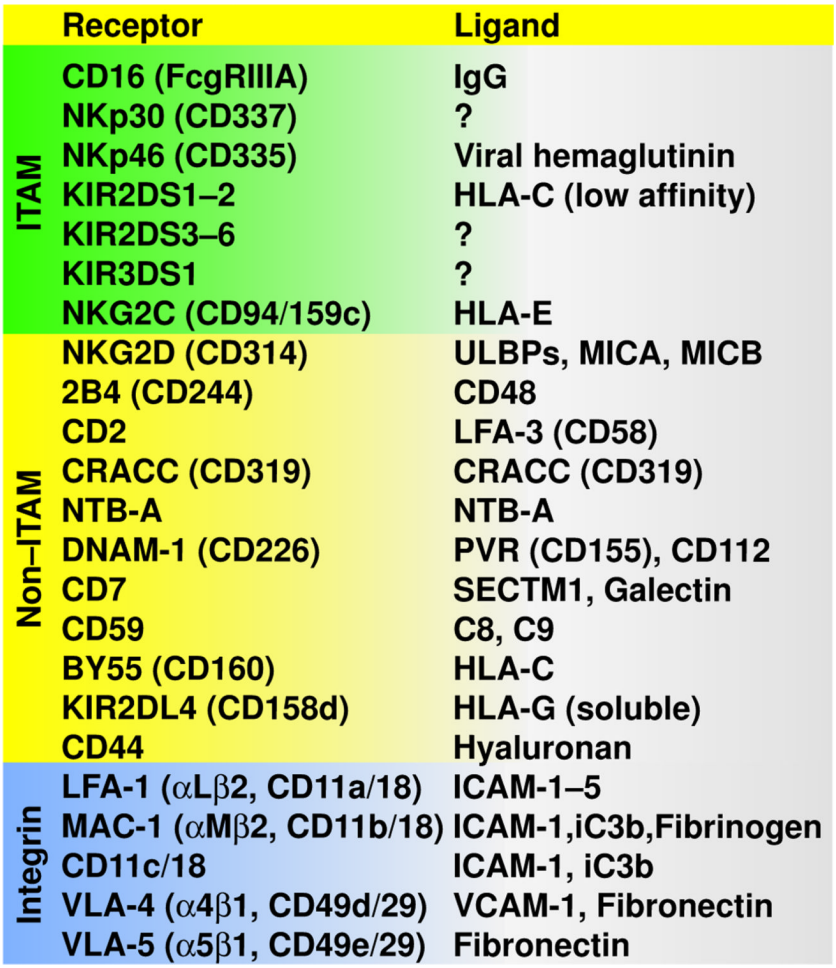

Fig. 2. Activating receptors expressed on human peripheral blood NK cells

Activating receptors expressed by freshly isolated, resting NK cells and their ligands are listed. KIR, NKG2C, and CD2 are only expressed by subsets of NK cells.

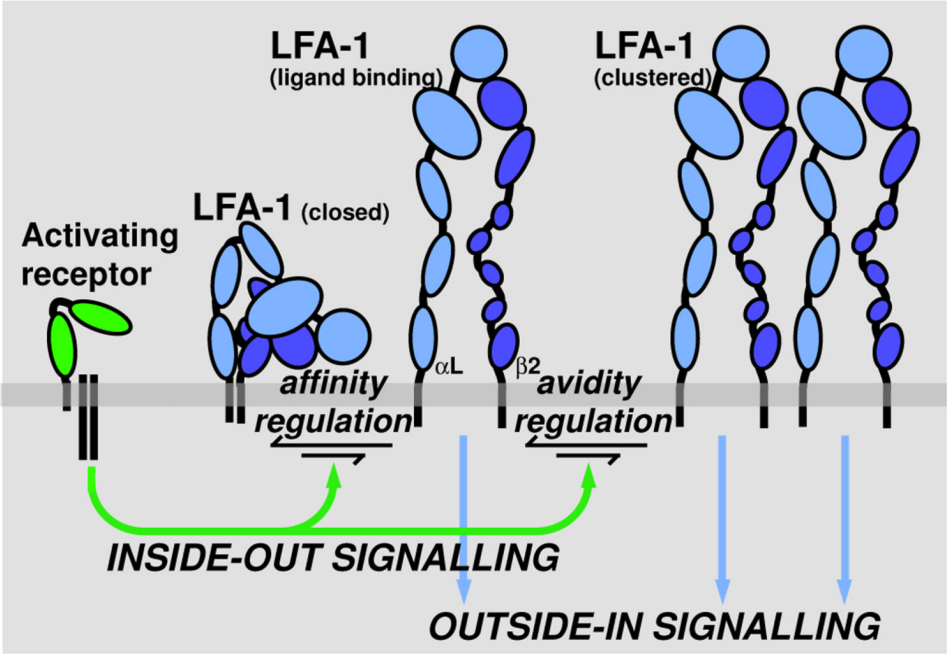

Fig. 3. Regulation of LFA-1–mediated adhesion

Inside-out signals from NK cell activating receptors may promote conformational changes leading to a high affinity, ligand binding conformation of LFA-1. They may also promote LFA-1 avidity through signals for clustering of LFA-1. Upon ligand binding, LFA-1–mediated outside-in signals are conveyed into the cell.

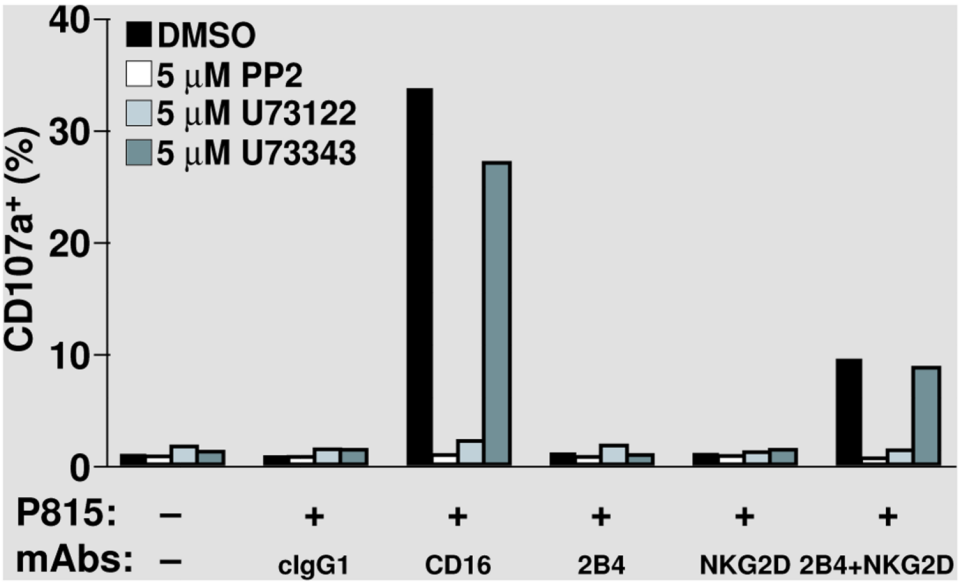

Fig. 4. Regulation of granule polarization

In resting NK cells, (A) LFA-1 alone or (B) a synergistic combination of signals from CD16 and 2B4 promote granule polarization.

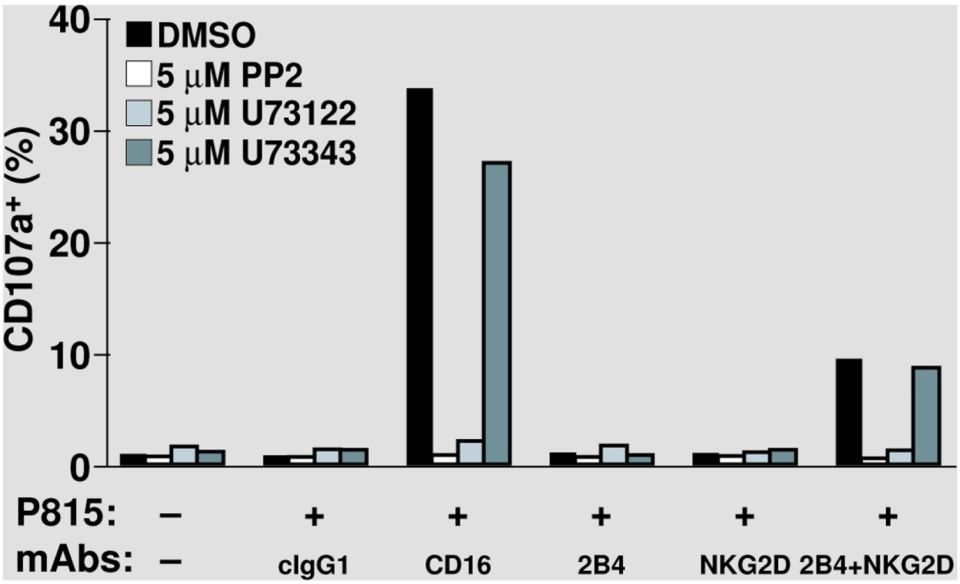

Fig. 5. Pharmacological inhibitors of PLC-γ abrogate degranulation by resting NK cells

Resting NK cells were pre-incubated with vehicle (DMSO), a Src kinase inhibitor (PP2), a PLC-γ inhibitor (U73122), or an inactive analog of the PLC-γ inhibitor (U73343), for 30 min. Thereafter, NK cells were incubated for 2 hours either alone or with P815 cells and mAbs in addition to inhibitors as specified. Cells were stained with flourochrome-conjugated anti-CD56 and anti-CD107a mAbs and analyzed by flow cytometry. NK cells were gated on forward scatter/side scatter plots and the percentage of $CD56^+$ $CD107a^+$ NK cells was calculated. One representative experiment is shown.

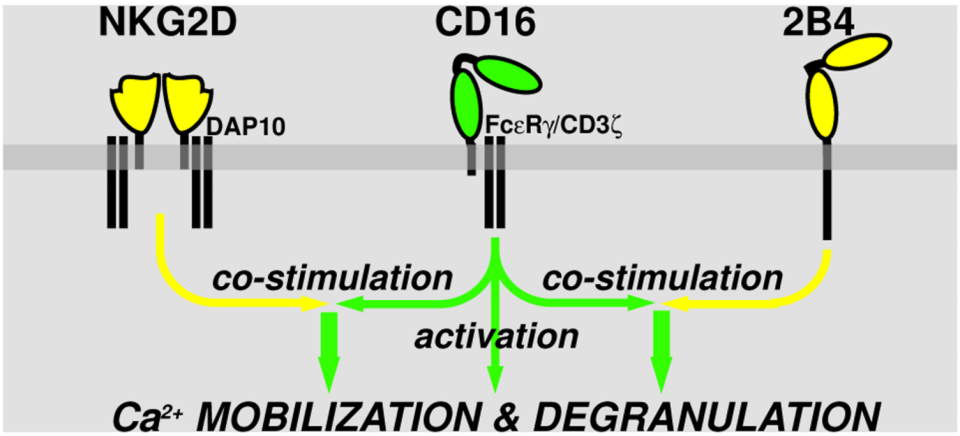

Fig. 6. Summary of NK cell activation by insect cells expressing ligands for human receptors

Ligands expressed on insect SC2 cells are indicated in yellow (CD48), blue (ICAM-1), and green (IgG). The outcome of interaction with NK cells is listed on the right.

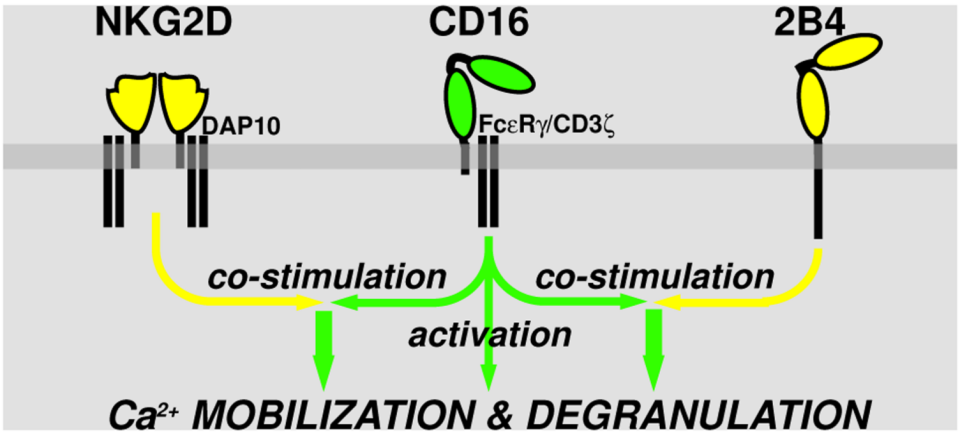

Fig. 7. Co-activation receptors co-stimulate degranulation for antibody-dependent cellular cytotoxicity

Engagement of CD16 is sufficient to induce Ca^{2+} mobilization and degranulation in resting NK cells. Ca^{2+} mobilization and degranulation is enhanced by co-engagement of co-stimulatory receptors such as NKG2D and 2B4.

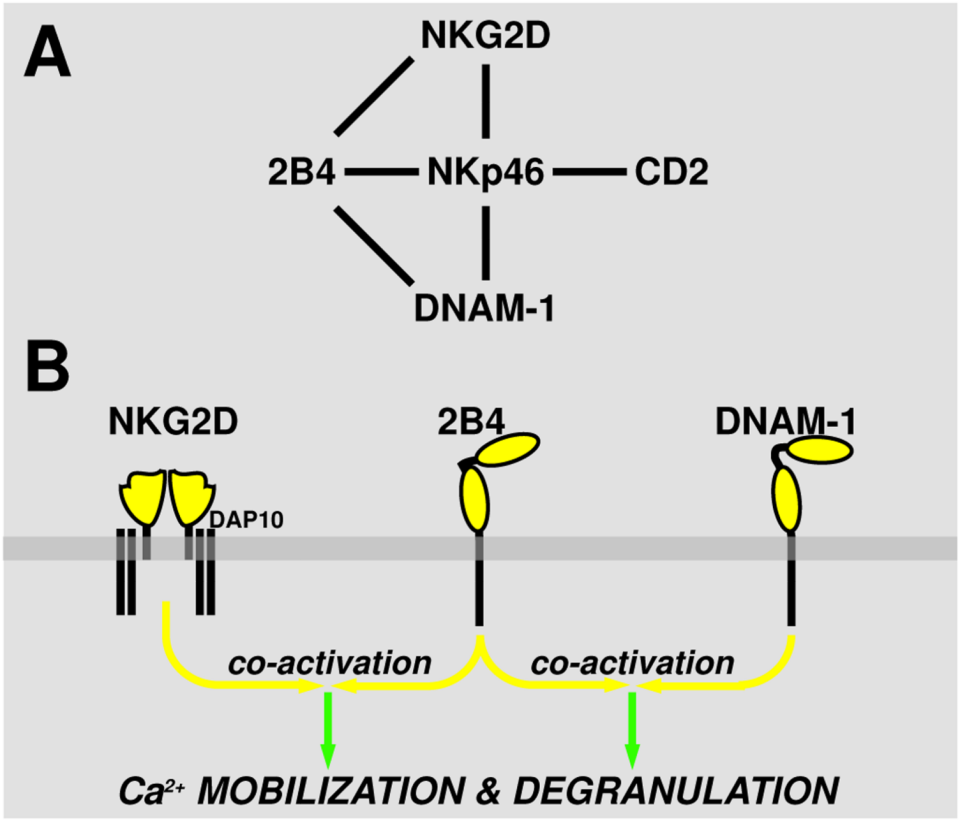

Fig. 8. Co-activation of resting NK cells

(A) Schematic representation of synergies among co-activation receptors for Ca^{2+} mobilization among receptors expressed on resting NK cells. (B) Co-engagement on non-ITAM–associated receptors can synergistically induce Ca^{2+} mobilization and degranulation in resting NK cells.

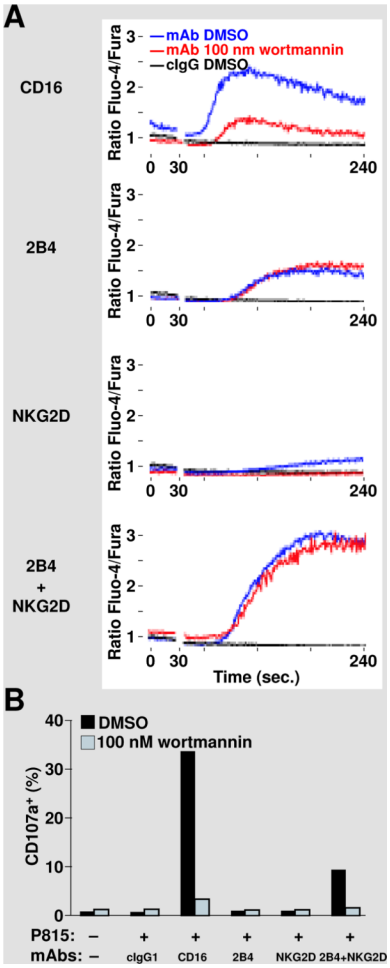

Fig. 9. Pharmacological inhibition of PI3K abrogates NKG2D–mediated co-activaiton of degranulation, but not Ca^{2+}-flux in resting NK cells

(A) NK cells were pre-incubated on ice with inhibitor and mAbs to the receptors indicated on the left, loaded with Fluo-4 and Fura Red, resuspended in HBSS 1% FBS, and pre-warmed at 37°C in the presence of inhibitor as indicated. Cells were analyzed by flow cytometry. After 30 seconds, secondary $F(ab')_2$ goat anti-mouse IgG was added to each sample. Changes in Fluo-4 (FL-1)/Fura (FL-3) ratios are shown as a function of time. Black lines represent activation with isotype control mAb; blue lines represent activation by receptors in the presence of vechicle (DMSO); red lines represent activation by receptors in the presence of PI3K inhibitor (wortmannin). (B) NK cells were pre-incubated with vehicle (dark bars) or inhibitor (shaded bars), and incubated for 2 hours either alone or with P815 cells and the indicated mAbs. Cells were stained with fluorochrome-conjugated anti-CD56 and anti-CD107a mAbs, and analyzed by flow cytometry. NK cells were gated on forward scatter/side scatter, and the percentage of $CD56^+$ $CD107a^+$ NK cells was calculated. One representative experiment is shown.