Published in final edited form as:
Annu Rev Immunol. 2013 ; 31: . doi:10.1146/annurev-immunol-020711-075005.

Controlling NK Cell Responses: Integration of Signals for Activation and Inhibition

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Abstract

Understanding how signals are integrated to control NK cell responsiveness in the absence of antigen-specific receptors has been a challenge, but recent work has revealed some underlying principles that govern NK cell responses. NK cells use an array of innate receptors to sense their environment and respond to alterations caused by infections, cellular stress and transformation. No single activation receptor dominates; instead, synergistic signals from combinations of receptors are integrated to activate natural cytotoxicity and cytokine production. Inhibitory receptors for MHC class I have a critical role in controlling NK cell responses and paradoxically, in maintaining NK cells in a state of responsiveness to subsequent activation events, a process referred to as licensing. MHC-I specific inhibitory receptors both block activation signals and trigger signals to phosphorylate and inactivate the small adaptor Crk. These different facets of inhibitory signaling are incorporated into a revocable license model for the reversible tuning of NK cell responsiveness.

Keywords

inhibitory receptor; licensing; major histocompatibility complex; natural killer; signaling

INTRODUCTION

Natural killer (NK) cells are lymphocytes that function at the interface between innate and adaptive immunity. NK cells contribute directly to immune defense through their effector functions, such as cytotoxicity and cytokine secretion, and indirectly by regulating antigen-presenting cells (APC) and the adaptive responses of T cells. Despite a lack of receptor diversity generated by DNA rearrangement, as used by B cells and T cells, NK cells share some properties with cells of the adaptive immune system. NK cells have the capacity to distinguish diseased cells from healthy cells, to mount powerful antiviral responses, and to maintain a pool of long-lived cells that had expanded during the response. One of the fundamental questions in NK cell biology is how specificity of responses can be achieved during interactions of NK cells with other cells and how healthy cells are spared from NK

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cell attack. Explorations into these issues have not only revealed features unique to NK cells, but have also contributed to our understanding of fundamental processes, such as cellular cytotoxicity and regulation of cellular responses by dominant inhibitory receptors, that occur in many other cellular and biological contexts.

This review will focus mainly on the regulation of NK cell responses through signals delivered by receptors that bind extracellular ligands, with an emphasis on signaling induced by contact with other cells (**Figure 1**). Rather than covering an exhaustive list of receptors and their associated signaling components, which have been reviewed previously (1-4), we will focus our attention on essential features of signal transduction for the regulation of NK cell responses and propose some general principles. The main points we wish to convey are:

- NK cells have to integrate signals received from multiple, germ line-encoded receptors in order to sense their environment and respond appropriately. The engagement of many different receptors during NK cell contact with target cells raises the question of how various signals are coordinated and how specificity can be achieved. Is there redundancy or a hierarchy among receptors for NK cell activation? Are receptors acting independently of each other? Recent work suggests that there is no dominant receptor for activation. Rather than forming a hierarchy, receptors combine into synergistic pairs to induce activation. Redundancy may occur within and among synergistic combinations of receptors, allowing NK cells greater flexibility in sensing and responding quickly to changes in their environment.
- The cytolytic activity of NK cells is similar to that of $CD8^+$ cytotoxic T lymphocytes (CTL). It occurs by polarized release of the contents of lytic granules toward target cells. However, NK cells differ from T cells in that the signals that control granule polarization and degranulation can be uncoupled, as they are not orchestrated through a central activation complex such as the T cell receptor (TCR). There is a strict requirement in NK cells for complementation of signals that trigger polarization and of those for degranulation.
- Another striking difference with T cells is that the β_2 integrin LFA-1 can signal autonomously in NK cells. In T cells, “inside-out” signals from the TCR or from chemokine receptors are required to activate LFA-1 into a ligand-receptive conformation. Not only does LFA-1 bind to ligand and signal independently of inside-out signaling in NK cells, it is also sufficient to induce granule polarization in these cells.
- Control of NK reactivity by inhibitory receptors specific for major histocompatibility complex class I molecules (MHC-I) is well established. But the regulation of NK cell responses is not due to a simple balance of activation and inhibition. As negative signals from inhibitory receptors tend to be dominant, the functional outcome of engaging both activation and inhibitory receptors is tilted in favor of inhibition. Inhibitory receptors for MHC-I carry immunoreceptor tyrosine-based inhibition motifs (ITIM) in their cytoplasmic tail, which become phosphorylated upon receptor engagement and recruit the tyrosine phosphatases SHP-1 and SHP-2. What are the specific signals targeted by SHP for inhibition?
- Another function recently attributed to inhibitory receptors for MHC-I is that they confer intrinsic responsiveness to NK cells, a property that has been referred to as “licensing” (5). Licensing is ITIM-dependent and requires the presence of an MHC-I ligand for the inhibitory receptor. The NK cell responsiveness dictated by inhibitory receptors is not fixed but can be tuned up or down through NK cell adaptation to the MHC-I environment. A major question is how licensing for

responsiveness is achieved through signaling by inhibitory receptors. Are ITIM-bearing receptors capable of transmitting signals other than tyrosine phosphatase-dependent dephosphorylation?

- The function of NK cells is both mediated and controlled by soluble factors, including chemokines, cytokines, and other secreted ligands of NK cell receptors (**Figure 1**). In this context, we will focus only on a few aspects that are unique to NK cells. In response to soluble HLA-G, an unusual killer cell immunoglobulin-like receptor (KIR) with Toll-like receptor (TLR)-like properties signals from endosomes for a proinflammatory and proangiogenic response. Since HLA-G expression is restricted to fetal trophoblast cells that invade the uterus soon after implantation, this activation pathway could contribute to vascular remodeling in early pregnancy.

Signaling by NK cell receptors has to be understood within a functional and biological framework. The biological functions performed by NK cells fall within three major categories.

NK cells Contribute to Defense Mechanisms that Control Infections and Establishment of Tumors

NK cells have an essential role in the control of infections by certain viruses, in particular those in the Herpes family, such as cytomegalovirus (CMV). NK cells are recruited to the site of infection by chemokines and perform their effector functions through perforin-dependent cytotoxicity and IFN-γ secretion (6). NK cells cannot clear the virus but are essential for controlling virus titers until an adaptive T cell response eliminates infected cells. NK cells also contribute to the control of parasitic infections, mostly through IFN-γ production (7). Antitumor activity of NK cells occurs through upregulation of ligands for NK activation receptors and/or loss of MHC-I on tumor cells. Mouse models with selective deletion of NK activation receptors have demonstrated an important contribution of NK cells to tumor immunosurveillance (8, 9).

NK cells Maintain Homeostasis in the Lymphoid System

While NK cells can promote inflammation by secreting IFN-γ and tumor necrosis factor (TNF)-α, they also control inflammation associated with immune responses by killing APCs and activated T cells (10). This is evident in familial hemophagocytic lymphohistiocytosis (FHL) patients, who have deficiencies in cellular cytotoxicity of lymphocytes. The lack of NK cytotoxic function in FHL patients is manifested by systemic inflammation. By their ability to dampen immune responses, NK cells may provide protection in the context of autoimmunity, such as multiple sclerosis (11, 12).

NK cells have a Role in Reproduction

NK cells are the predominant lymphocyte subset in the uterus (13). They interact with fetal trophoblast cells that invade the maternal uterine tissue upon implantation and contribute to the remodeling of the maternal vasculature. Genetic associations between the maternal repertoire of killer cell Ig-like receptors (KIR) and MHC-I expressed by the fetus have shown that combinations favoring NK cell activation are beneficial, whereas combinations that promote inhibition correlate with pregnancy complications such as preeclampsia (14).

INTEGRATION OF SIGNALS FOR ACTIVATION

The multitude of receptors capable of triggering NK cell responses, such as cytokine secretion and direct cellular cytotoxicity (**Figure 1**), is potentially dangerous. How are their signals kept in check to avoid inappropriate responses against healthy cells? Control of NK

cell reactivity is exerted at two levels: expression of ligands for these activation receptors, and of MHC-I ligands for inhibitory receptors. But what are the minimal requirements for activation of NK cell effector function? Given the many receptor--ligand interactions that take place during NK--target cell contacts, it is difficult to determine the contribution of individual receptors. A reductionist approach using reconstituted systems was essential to tease apart contributions from different receptors (15-17). The use of Abs to selectively crosslink specific receptors bypasses the complexity of a mammalian target cells but does not reproduce physiological receptor--ligand interactions. The ligand of a given receptor can be expressed on insect cells or attached to artificial lipid bilayers and tested for its ability to induce signals in NK cells. However, a limitation has been that the ligands for several of the NK activation receptors have yet to be identified. Recent data acquired with primary, unstimulated NK cells has led to a new appreciation of the regulation of NK cell activation. Apart from the Fc receptor FcγRIIIa (CD16), other receptors, including NKG2D (CD314) and NKp46 (CD335), do not activate on their own (17, 18). Activation of NK cells by any of the receptors tested required complementation with another receptor in order to obtain synergistic activation signals. Negative control of NK activation signals occurs at different levels, not only through inhibition by receptors for MHC-I. A revision of how we think about the regulation of NK cell activation is required to accommodate this new information.

An Abundance of Receptors for Activation

Our understanding of signaling by NK cell receptors is skewed towards a few, for which signaling pathways have been described, while less is known about the signaling properties of most of the other receptors. Among the receptors considered dominant are those associated with immunoreceptor tyrosine-based activation motif (ITAM)-bearing signaling molecules (e.g., NKp46, which is associated with the FcR γ chain or the TCR ζ chain), and the receptor NKG2D, which is associated with the signaling molecule DAP10 (19, 20). Receptors considered costimulatory include members of the signaling lymphocytic activation molecule (SLAM) family, such as 2B4 (CD244), as well as unrelated receptors such as DNAM-1 (CD226), CD2, and NKp80 (product of the *KLRF1* gene). We will not review each receptor in detail but will highlight recent work on their signaling properties and outline some general principles that govern activation of NK cell functions.

Receptors associated with ITAM-bearing molecules—Three ITAM-bearing molecules contribute to signaling by a number of different activation receptors on NK cells. The FcR γ and TCR ζ chains form homodimers and heterodimers that associate with CD16. Among the three natural cytotoxicity receptors (NCR), NKp46 and NKp30 associate with FcR γ and/or TCR ζ, while NKp44 is associated with the signaling adaptor DAP12 (19). DAP12 carries a single ITAM and forms a homodimer (21, 22). Ubiquitously expressed, DAP12 is found associated with several other receptors in multiple cell types. Signaling through ITAMs has been studied in great detail, as it is the signaling pathway used by several of the major immunoreceptors, such as TCR (23). The two tyrosines in the ITAM are phosphorylated by Src-kinase family members, and phosphorylated ITAMs form a binding site for the Src-homology domain 2 (SH2) domains of the ZAP70 and Syk tyrosine kinases.

The only transmembrane protein, normally expressed at the plasma membrane, that has been identified as a ligand for an NCR is B7-H6, which binds to NKp30 and is expressed on several tumor cell lines (24). The ability of B7-H6 to activate NK cells on its own has not been tested. NKp30 is involved in the activation of NK cells by dendritic cells (DC) (25). Even though NKp46 is associated with ITAM-bearing subunits, stimulation of primary resting NK cells with NKp46 Abs was not sufficient to activate degranulation (18). However, when combined with signals from any one of the receptors 2B4, DNAM-1, NKG2D or CD2, NKp46 induced degranulation. This requirement for a synergistic

combination of activation receptors may serve as a safeguard to prevent unrestrained activation of NK cells. This stands in contrast to signaling by CD16, which is sufficient to activate degranulation. Through binding to the Fc portion of Abs, CD16 endows NK cells with the ability to detect cells coated with Abs and to eliminate them by Ab-dependent cellular cytotoxicity (ADCC). In this case, specificity is determined by adaptive, Ab-producing B cells, which could be the reason why activation of NK cells by CD16 is not subject to the requirement of synergy with other receptors.

The KIR and CD94-NKG2 families of inhibitory receptors include members that are activating, due to their association with DAP12 (20, 26). The activating isoforms of the KIR family appear to have evolved more rapidly than inhibitory KIRs, perhaps by selection imposed by pathogens (27, 28). Genetic studies have revealed that certain activating KIRs, in combination with specific MHC-I ligands, may provide protection from progression to AIDS in HIV-infected individuals (29), and from pre-eclampsia in pregnant mothers (30). A difficulty in understanding the basis of the protective effect is that ligands for most of the activating KIRs have not been identified.

An unusual activating KIR with a single ITIM and the ability to associate with the ITAM-containing FcR γ chain is CD158d (KIR2DL4) (31, 32). While it is capable of triggering weak cytotoxicity from the cell surface, most of the receptor resides in endosomes and signals from that site. CD158d signals in transfected 293 cells by a pathway that is independent of both the ITIM and the arginine in the transmembrane domain, which is required for association with the FcR γ chain (33).

In mice, the function performed by KIRs in humans is assigned to the Ly49 receptors, which are C-type lectins encoded in the NK gene complex (34). Like the KIR genes, the Ly49 family is highly polymorphic and multigenic. Ly49 members are expressed as dimers, with activating isoforms of Ly49 pairing with DAP12, and inhibitory isoforms carrying an ITIM in their cytoplasmic tail. Ly49H and Ly49P are activating forms expressed in specific Ly49 haplotypes, which detect cells infected with mouse CMV (MCMV) and provide resistance to infection (35-38). By analogy with the Ly49-mediated resistance to MCMV, the expansion of human NK cells that express the activating receptor CD94-NKG2C during CMV and Hantavirus infections suggests that such NK cells may provide protection (39-42).

NKG2D (CD314)—NKG2D binds to a number of ligands that are induced on cells that are under stress due to infection, transformation or DNA damage (43). These include MICA and MICB and the family of ULBP molecules in humans, and H60 and Mult1 and the family of RAE-1 molecules in mice (44). Therefore, it has an important function in targeting NK cell responses towards abnormal cells (45). Mice deficient in NKG2D are more prone to spontaneous tumors (8). NKG2D signals through its association with the short transmembrane molecule DAP10, which carries a tyrosine-based motif (YxxM) different from the ITAM (46). Phosphorylated DAP10 binds either the p85 subunit of phosphoinositide 3-kinase (PI3K) or the small adaptor Grb2 in association with the guanine exchange factor Vav1 (47-50). Stimulation of NK cells through NKG2D results in binding of the small adaptor CrkL to the p85 subunit of PI3K. CrkL contributes to NKG2D signaling for adhesion, granule polarization toward the target cell, and degranulation (51). As CrkL binds to guanine nucleotide exchange factors (GEF) that activate the GTPases Rac1 and Rap1, it can promote synapse formation and LFA-1--dependent adhesion.

Receptors of the SLAM family—One of the best characterized NK cell activation receptors is 2B4 (CD244), which is a member of the SLAM receptor family (3). The immunoglobulin (Ig)-like receptors of the SLAM family are expressed on hematopoietic cells. They mediate cell--cell interactions through homophilic binding (i.e., they bind to

themselves in *trans*), except for 2B4, which binds to another Ig-like hematopoietic molecule, CD48. NK cells express every member of the SLAM family of receptors, except for SLAM (CD150, *SLAMF1*) itself. In addition to 2B4, they are natural killer, T and B cell antigen (NTB-A, *SLAMF6*, Ly108 in mouse), CD2-like receptor activating cytotoxic cells (CRACC, CD319, *SLAMF7*), Ly9 (CD229), and CD84 (52). All share tyrosine-based motifs in their cytoplasmic tails, S/TxYxxL/I, which are similar to ITIMs and referred to as immunoreceptor tyrosine-based switch motifs (ITSM) (53). These receptors transmit activation signals through the SLAM-associated protein (SAP, encoded by the gene *SH2D1A*), which recruits the tyrosine kinase Fyn (54). Mutations in *SH2D1A* form the genetic basis of X-linked lymphoproliferative (XLP) disease (55, 56). SAP controls 2B4-dependent NK cell activation in two ways: by Fyn-induced phosphorylation of Vav1, and by blocking recruitment of the inhibitory SH2 domain-containing inositol 5'phosphatase-1 (SHIP-1), which dampens Ca^{2+} influx (57). Inhibitory molecules recruited in the absence of SAP include SHIP, SHP-1 and SHP-2, and Csk (58, 59). NK cells express another adaptor related to SAP, called Ewing's sarcoma-associated transcript-2 (EAT-2), which does not bind Fyn (60). SAP and EAT-2 combine to promote NK cell activation in response to CD48-expressing cells (61), and in NK cells from the double SAP/EAT-2 deficient mice, 2B4 was even more inhibitory than in the absence of SAP (57).

An inhibitory function of 2B4 occurs not only in SAP mutants, but also in cells from normal mice. The inhibitory function of 2B4 is important for the protection of activated $CD8^+$ T cells from NK cells during LCMV infection of mice. In 2B4 deficient mice, NK cells killed activated $CD8^+$ T cells early in infection, leading to viral persistence and worsened pathology (62). How the balance between the activating and inhibitory functions of 2B4 is set in normal, SAP^+ individuals, and whether it is regulated at the level of SAP expression is not known.

Other activating receptors—NK cells express a long list of other receptors with activation potential, such as DNAM-1 and NKp80. For most of them, limited information on their signaling properties is available. DNAM-1 has an essential role in preventing spontaneous tumor formation and in controlling tumor growth (9, 63-66). DNAM-1 binds to the poliovirus receptor CD155 and the nectin adhesion molecule CD112 (67), which are upregulated on tumor cells. DNAM-1 participates, together with NKp30, in the killing of dendritic cells (68). Protein kinase C (PKC)-dependent phosphorylation of Serine 329 in DNAM-1 promotes ligand binding (69). DNAM-1 has been found associated physically and functionally with LFA-1 and the tyrosine kinase Fyn (70).

NKp80 and NKp65, which have activating properties in NK cells, share common structural and genetic features: a tyrosine sequence motif in their N-terminal cytoplasmic tail that corresponds to half of an ITAM (a hemi-ITAM, or hemITAM), and tight linkage of their genes with the genes encoding their ligands. NKp80 binds to activation-induced C-type lectin (AICL, encoded by *CLEC2B*), which is upregulated by TLR stimulation on myeloid cells (71). NKp65 binds to keratinocyte-associated C-type lectin (KACL, encoded by *CLEC2A*), which is expressed exclusively in the skin, and stimulates cytotoxicity and cytokine release by NK cells (72). NKp80 stimulates Syk phosphorylation and Syk-dependent cytotoxicity (73). Signaling through a hemITAM by recruitment of the tyrosine kinase Syk was first described for the receptor Dectin-1 (*CLEC7A*) expressed in myeloid cells (74). The Syk-dependent signal induced by Dectin-1 leads to activation of the NLRP3 inflammasome and to a NF-κB-dependent proinflammatory response (75). The signals downstream of Syk after stimulation by NKp80 are not known, and it is not clear whether hemITAM signaling in NK cells will have the same outcome as in myeloid cells.

Many other receptors can contribute to NK cell activation, including CD2, CD44, CD137, fractalkine receptor CX3CR1, TNF receptor family member CD27, and CD160. Much work remains to be done in determining how and when the various NK cell receptors deliver signals for activation.

Redundancy and specificity of components in the NK cell signaling toolbox— NK cells tend to express several members of a given family of signaling molecules, which are otherwise expressed selectively in one cell type. For example, NK cells express the T cell-specific tyrosine kinase ZAP70 and phospholipase C (PLC)-γ1, as well as the B cell-specific tyrosine kinase Syk and PLC-γ2. This redundancy may explain why NK cell function often remains normal in mice with targeted deletions of genes encoding signaling molecules. Furthermore, a redundancy of activation pathways in NK cells endows them with the ability to detect different types of target cells and to mount different types of responses. It is now clear that NK cells can kill target cells through ITAM-dependent and ITAM-independent pathways. The original observation that NK cells lacking both ZAP70 and Syk kinases have normal cytotoxic activity toward sensitive target cells, implying that ITAM-based pathways are dispensable for natural cytotoxicity toward certain target cells, was initially a surprise (76).

Two signaling molecules that are critical for NK cell activation are Vav1 and PLC-γ2. NK cells in Vav1-deficient mice are not as impaired as T cells but show defects in tumor cell killing (77). NK cells from PLC-γ2-deficent mice have defects in Ca^{2+} mobilization, degranulation, cytotoxicity, and in the control of MCMV infection (78, 79). Furthermore, PLAID (PLC-γ2–associated antibody deficiency and immune dysregulation) patients with mutations in *PLCG2* show defective NK cell degranulation after incubation with sensitive target cells and defective Ca^{2+} mobilization after stimulation through NKG2D and 2B4 (80). In line with the cell type-specific expression of PLC-γ2, defects in Ca^{2+} mobilization were also evident in BCR-stimulated B cells, but not after crosslinking of TCR on T cells (80).

NKG2D couples preferentially to PLC-γ2, whereas ITAM-based pathways can use either one of the two PLC-γ isoforms (81). Furthermore, NKG2D signals selectively through Vav1, while signaling through the ITAMs of the FcR γ chain or DAP12 requires Vav2 and Vav3 (82). Thus, there are examples of parallel, redundant pathways that are distinguished by the use of dedicated effector molecules. Signaling by NK cell activation receptors often leads to degranulation and to cytokine secretion. The downstream signaling requirements for these two responses are different, even if triggered by the same receptor. For example, PKC-θ, a member of the novel PKC family, is required for ITAM-dependent transcriptional IFN-γ responses but not for cytotoxicity (83).

Faced with this large apparent redundancy in activation receptors and signaling pathways that contribute to stimulation of NK cells, an important question is how NK cells coordinate and integrate these different signaling inputs.

Synergistic Combinations of Coactivation Receptors

Is there redundancy, hierarchy or complementation among the multiplicity of receptors on NK cells that can deliver activation signals? Is any receptor sufficient and is any receptor necessary to trigger cytotoxicity, cytokine secretion, or other NK cell responses? Receptors that are generally considered “activating” (i.e., sufficient to induce a functional response) include NKG2D and the three members of the NCR family, NKp46, NKp44, and NKp30 (19, 20). Expression of ligands for NKG2D on cells that are normally resistant to NK cytotoxicity can result in target cell lysis (84). Early on, such results were taken to mean that NKG2D was sufficient to activate natural cytotoxicity and, further, that it may be resistant to inhibition by receptors for MHC-I. Lysis of many tumor cell lines or fresh tumor cells can

be blocked with Abs to one or more of the NCRs (19), showing that these receptors are necessary to activate cytotoxicity. But are they sufficient? Most of the other NK receptors that signal for activation, such as DNAM-1, 2B4, and CD2, have been typically considered to be costimulatory or contributors to adhesion and accessory functions.

The best way to determine the precise role of any given receptor on NK cells is to test each one individually, or in combination with others. Abs provide a convenient tool to selectively crosslink receptors, but they do not even approximate the biophysical properties of receptor--ligand interactions in opposing membranes. More physiological conditions can be achieved by expression of ligands for NK cell receptors on *Drosophila* insect cells, or by attaching ligands to artificial supported lipid bilayers. A number of unexpected results were obtained when NK cells were presented with specific ligands using such a reductionist approach.

Crosslinking NK receptors with Abs showed that CD16 was the only receptor tested that was sufficient to activate degranulation in primary resting, human NK cells (18). The other receptors, NKp46, 2B4, NKG2D, DNAM-1, and CD2 stimulated degranulation only in combination with others. Some, but not all pairwise combinations of receptors result in synergistic activation. Therefore, NK cell "activation" receptors, including NKp46 and NKG2D, do not stimulate degranulation on their own. These results were confirmed using the *Drosophila* insect cell line S2, transfected to express ligands of NK receptors, either alone or in combination, and S2 cells coated with an anti-S2 polyclonal rabbit IgG to stimulate CD16 (17). Insect cells stimulated degranulation and were killed by ADCC, or when pairs of ligands for synergistic combinations of receptors were coexpressed. For example, CD48 (2B4 ligand) and ULBP1 (NKG2D ligand), or CD48 and CD155 (DNAM-1 ligands), but not ULBP1 and CD155, induce NK cell degranulation (17). The hierarchy in NK cell activation receptors, in the context of primary, resting NK cells, has to be revised. First, no single receptor for natural cytotoxicity, among those tested, is sufficient to induce degranulation. (CD16 is not a natural cytotoxicity receptor, as it mediates ADCC, the specificity of which is determined by B cells of the adaptive immune system.) Second, receptors thought to be accessory or costimulating, such as DNAM-1 and 2B4, have gained equal status with NKG2D and NCRs. All are "coactivation" receptors. Third, natural cytotoxicity can occur in the absence of ligands for NKG2D and for NCR, as with the combination of 2B4 and DNAM-1.

Receptor synergy for secretion of cytokines and chemokines—The stimulation of cytokine and chemokine secretion by NK cell receptors that bind to ligands expressed on other cells (as opposed to receptors for soluble molecules) is more complex than the degranulation response (85). For example, 2B4 is sufficient to induce IFN-γ secretion on its own. In combination with a synergistic partner receptor, 2B4 signals for greater IFN-γ production and also for secretion of additional cytokines and chemokines. Both the magnitude of cytokine secretion and the complexity of the secretory profile increase when different coactivation receptors are coengaged (85). Another conclusion drawn from the stimulation of NK cells by combinations of receptor ligands for NK receptors is that the distinction between the two major subsets of peripheral NK cells is not as previously thought. The majority of NK cells in peripheral blood are $CD56^{dim}CD16^{+}$. The $CD56^{bright}CD16^{-}$ NK cells, which constitute the smaller subset in peripheral blood, are more abundant in tissues and are stronger cytokine/chemokine producers when stimulated with soluble factors, such as IL-12 and IL-18 (86). However, the cytokine/chemokine secretion induced by contact with target cells is greater with $CD56^{dim}CD16^{+}$ than with $CD56^{bright}CD16^{-}$ cells (85). Thus, the distinction is not about which subset makes more cytokine or kills better, but rather that one subset, the $CD56^{bright}$, responds better to soluble

factors, while the other, the $CD56^{dim}$, responds better to receptors that bind to ligands anchored on other cells.

The molecular basis for synergy among coactivation receptors—It is somewhat puzzling that several receptors with very different signaling properties can synergize in various combinations for NK cell activation. This suggests that signals converge toward a critical control element and that a certain activation threshold has to be reached. However, synergy is best achieved through complementation of independent signals, rather than by a simple increase in signal input. With the combination of receptors 2B4 and NKG2D, either crosslinked with Abs or coengaged with target cells coexpressing their ligands, synergy was detected at the level of PLC-γ phosphorylation and phosphorylation of the mitogen-activated protein kinase (MAPK) Erk (87). Phosphorylation of Vav1, on the other hand, occurs after engaging each receptor individually, and is additive after coengagement of both receptors. Vav1 is required for natural cytotoxicity induced by coactivation receptors. The requirement for receptor synergy in this Vav1-dependent pathway is due to inhibition by the ubiquitin ligase c-Cbl, which is overcome by signals from two synergistic receptors, such as 2B4 with NKG2D, and 2B4 with DNAM-1 (87).

The additive, rather than synergistic phosphorylation of Vav1 after coengagement of 2B4 with either NKG2D or DNAM-1 is compatible with either a requirement for a threshold of activated Vav1, or with complementation of different pools of Vav1. The answer is that synergy involves the selective phosphorylation of two tyrosine residues on the adaptor molecule SLP-76 (88). SLP-76 is required for the synergistic activation of human NK cells by 2B4 and NKG2D and 2B4 with DNAM-1 (88). In mouse NK cells, SLP-76 is also required for NKG2D-dependent activation, but less so for killing of the sensitive YAC-1 target cells (89, 90). Redundant signaling pathways induced by YAC-1 cells may mask the requirement for SLP-76 in a given pathway. Two tyrosines, at position 113 and 128 in SLP-76, are phosphorylated in T cells after stimulation through the TCR (91), or in NK cells stimulated by CD16. In contrast, 2B4 signaling leads to preferential phosphorylation at Tyr113, while NKG2D signals for phosphorylation at Tyr128 (**Figure 2**). Each one of these two phospho-tyrosines can bind Vav1 and the adaptor Nck. Vav1 and Nck also bind each other, such that a $(Vav1)_2$:$(Nck)_2$:SLP-76 stoichiometric complex can be formed (92). The dependence of synergistic activation on complementary phosphorylation of two tyrosines in SLP-76 is further supported by the synergistic combination of activation through 2B4 and DNAM-1. Engagement of DNAM-1, which does not synergize with NKG2D, results in phosphorylation at the same Tyr128 (88). How recruitment of two Vav1 molecules by one SLP-76 molecule, or a complementation of separate Vav1--pY113-SLP-76 and Vav1--pY128-SLP-76 complexes, results in synergistic signaling is not known. Transactivation of adjacent Vav1 molecules or the protection of $(Vav1)_2$--SLP-76 complexes from c-Cbl-dependent ubiquitylation are possibilities (**Figure 2**).

Uncoupling of Signals for Degranulation and Granule Polarization

Target cells are killed by NK cells as a result of polarized release of the contents of lytic granules at the immunological synapse. Therefore, at least two important steps are required for cytotoxicity: movement of the microtubule organizing center (MTOC) and MTOC-associated granules toward the site at the plasma membrane that is in contact with target cells (polarization), and fusion of the granules with the plasma membrane (degranulation). Having these two steps under the control of different activation pathways would impose another requirement for synergy and add a safeguard against unrestrained NK cytotoxicity. In resting NK cells, ADCC induced by CD16 and synergistic signals induced by coactivation receptors in the absence of LFA-1 engagement promote degranulation but not polarization (17, 87). Conversely, LFA-1 binding to ICAM-1 induces granule polarization

but not degranulation (15, 16). Therefore, signals for granule polarization and for degranulation can be uncoupled. Signals for granule polarization are more stringent in mouse NK cells, and require coligation of LFA-1 and NKG2D (93). Signaling requirements to induce polarization are also different in IL-2--activated versus resting NK cells: In IL-2--activated human NK cells, NKG2D, as well as LFA-1, can signal on their own for granule polarization (94). Autonomous signaling by LFA-1 is a unique feature of NK cells, as integrins generally depend on signaling by other receptors to become activated.

Signal Integration at Immunological Synapses

Each of the two processes, granule polarization and degranulation, involves multiple intermediate steps (10), such as dynein-dependent transport of granules to the MTOC, docking of the MTOC at the plasma membrane, docking and priming of granules at, and fusion with the plasma membrane (95-99). NK cells are very well suited as a system to study cytotoxic immunological synapses. Unlike T cells, there is no requirement to isolate antigen-specific clones or to generate transgenic mice expressing a single antigen-specific TCR. NK cells also have some unique properties that are not shared with T cells. For example, weak TCR signals in CTL are sufficient to promote movement of the MTOC to the synapse, but not for efficient recruitment of granules to the MTOC (100). In contrast, movement of granules to the MTOC in NK cells is a fast, dynein-dependent and actin-independent process that precedes movement of the MTOC toward the synapse (95).

The small GTPase of the Rho family Cdc42 is a key control element in cell polarity (101). During NK cell--target cell interaction, Cdc42 is activated and its activity oscillates, as shown with a biosensor for GTP-bound Cdc42 (102). The p85 subunit of PI3K is required for Cdc42 activation, for the periodicity of Cdc42 oscillation, and for MTOC polarization, suggesting that Cdc42 oscillation contributes to granule polarization at cytotoxic synapses (102). Downstream of activated Cdc42, the Cdc42-interacting protein-4 (CIP4), which binds to microtubules, is required for MTOC polarization and cytotoxicity, but not for F-actin accumulation (103).

A late step in cytotoxicity is the movement of lytic granules through the cortical actin cytoskeleton toward the plasma membrane and this step is myosin IIA-dependent (104, 105). High resolution microscopy has revealed that NK cytotoxic synapses are not depleted of F-actin but that the remaining F-actin network includes a few openings just wide enough to allow passage of lytic granules, which are about 250--500 nm in diameter (106, 107). The precise signals that control each of the steps that guide granules first along microtubules toward the MTOC, and then from microtubules across an F-actin layer to the plasma membrane are not known.

Degranulation by NK cells can be triggered by CD16 alone or by synergy of coactivation receptors (16, 17, 108). It depends on the participation of many proteins, some of which have been tested directly for their role in NK cell degranulation. These include syntaxin 11, which is mutated in FHL4 patients (109, 110), dynamin 2 (111), and the two soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins VAMP4 and VAMP7 (112). VAMP7, but not VAMP4, is also involved in IFN-γ secretion (112). The proteins Rab27a, which is mutated in Griscelli syndrome type 2, and Munc13-4, which is mutated in FHL3, are required for a late step in granule docking and fusion in cytotoxic lymphocytes (113). Recruitment of these two molecules to perforin-containing granules in NK cells is controlled by different signals. LFA-1, NKG2D, and 2B4 each signal for Rab27a recruitment, whereas CD16 induces Rab27a-dependent recruitment of Munc13-4 to perforin compartments (99). How these signals are properly coordinated for functional cellular cytotoxicity is not known.

The noncytolytic immunological synapse between NK cells and DC has unique properties. NK--DC conjugates are stable, and F-actin accumulated at the synapse in the DC promotes clustering of MHC-I and protection from lysis by the NK cell (114). These properties may promote stimulation of NK cells by DC in the absence of cytotoxicity (115).

A Central Role for β_2 Integrin

NK cells provide a unique opportunity to examine the signaling properties of an integrin in the absence of "inside-out" signals. In primary T cells, the β_2 integrin LFA-1 remains in a closed conformation unless the TCR or a chemokine receptor transmits inside-out signals that trigger an extended conformation of LFA-1 (116). As LFA-1 does not signal on its own in T cells, it is not possible to dissect the relative contribution of TCR and LFA-1 signals to T cell responses. In primary CTLs, TCR signals induce a partial polarization of lytic granules toward the synapse. LFA-1 does not bind to ICAM-1 on its own, and does not signal in the absence of TCR engagement. However, the combination of both TCR and LFA-1 signals results in efficient granule polarization (117). This means either that TCR transmits inside-out signals to LFA-1, which then facilitates TCR-dependent granule polarization by promoting adhesion, or that TCR provides only inside-out signals and that the polarization is controlled mainly by LFA-1. These two interpretations cannot be distinguished. As inside-out signaling by other receptors for the binding of LFA-1 to ICAM-1 is not required in NK cells, one can examine the outcome of signaling by LFA-1 alone (15). (In NK cells, LFA-1 provides its own inside-out signal, which promotes a signal-dependent increase in binding of NK cells to ICAM-1 (118).) LFA-1 binding to purified ICAM-1 on beads or ICAM-1 expressed on insect cells is sufficient to induce granule polarization (15, 16).

The Santoni group has shown that β_1 and β_2 integrins in NK cells initiate signals that lead to phosphorylation of the kinase Pyk2 and its association with the scaffold protein paxillin (119, 120). Pyk2 is required for the reorientation of the MTOC and paxillin in NK cells that are in contact with sensitive target cells (121). Studies of T cell immunological synapses have shown that paxillin is bound to the MTOC and that the combined signals from LFA-1 and TCR result in paxillin recruitment to the site of integrin engagement (122). This is consistent with the role of paxillin in the LFA-1--dependent polarization of granules in NK cells (123).

Binding of LFA-1 on NK cells to purified ICAM-1 results in tyrosine phosphorylation of several molecules that are also phosphorylated downstream of the ITAM-dependent signaling by CD16, including PLC-γ and Syk (123). This is surprising, considering that LFA-1 does not induce Ca^{2+} mobilization and that CD16 does not induce polarization. How the signaling pathways diverge downstream of CD16 and LFA-1, one leading to degranulation, and the other to polarization, is still not known. In this respect, NK cells are also different from neutrophils, where β_2 integrin couples to ITAM-bearing subunits to signal for Ca^{2+} mobilization and degranulation (124, 125). It is not known how β_2 integrins signal through ITAM-bearing DAP12 or FcR γ chain in neutrophils.

The requirements for granule polarization differ in mouse NK cells. Binding of ICAM-1-coated beads to mouse NK cells is sufficient to induce actin cytoskeletal reorganization, but not polarization of lytic granules. Granule polarization in mouse NK cells requires signaling induced by ICAM-1 and coligated NKG2D (93). Talin, which is associated with the cytoplasmic tail of LFA-1 and required for inside-out signaling, is also required for LFA-1 outside-in signaling for polarization (93). Talin contributes to LFA-1 signaling in two ways: by recruitment of the Arp2/3 actin nucleating complex through vinculin, and by association with phosphatidylinositol 4-phosphate 5-kinase (126). Binding of ICAM-1--coated beads to LFA-1 is sufficient to induce an increase in PI-4, 5-phosphate (PIP_2) and recruit the

Wiskott-Aldrich Syndrome protein (WASp), which initiates Arp2/3-dependent actin polymerization (126).

Another critical role of LFA-1 in NK cells is in the organization of the cytotoxic immunological synapse. ICAM-1 is required for the formation of organized natural cytotoxicity synapses, including a peripheral ring of ICAM-1 around a central region where the CD48 ligand of 2B4 accumulates, as observed with artificial lipid bilayers carrying ligands for NK receptors (108). ICAM-1 is also required for the formation of a stable central zone at the synapse where active membrane internalization occurs and where exocytosed lysosome-associated protein 1 (LAMP-1) molecules are retrieved into an endocytic compartment, presumably for the recycling of lytic granule membranes (108). The unique ability of LFA-1 to signal on its own in NK cells has revealed a much greater signaling capability of this integrin than previously appreciated.

SIGNALS INDUCED BY SOLUBLE FACTORS

As circulating lymphocytes, NK cells are exposed to many different types of soluble activators, including chemokines, cytokines, and interferons that control NK cell survival, proliferation, migration, and their own production of soluble factors (**Figure 1**). Signaling by the receptors for these soluble molecules has been studied extensively in a number of cells, including NK cells, and falls beyond the scope of this review. A few aspects of signals induced by soluble factors relating to unique properties of NK cells are highlighted. First among them is that stimulation of NK cells by purely soluble molecules is not subject to the inhibition mediated by NK receptors that bind to MHC-I on other cells (**Figure 1**). This is because inhibition operates within the context of immunological synapses, blocks signaling by coclustered activation receptors, and does not render NK cells unresponsive to other stimuli (see next section on inhibition).

An interesting exception may be the stimulation of NK cells by IL-15, a cytokine essential for NK survival. IL-15 is not released as a free cytokine but is bound to the α chain of the IL-15 receptor (IL-15Rα) and is “trans-presented” by cells expressing the IL-15Rα--IL-15 complex at their surface to the intermediate affinity $\beta\gamma_c$ (CD122/CD132) receptor on T cells and NK cells (127-130). NK cells do not need to express IL-15Rα nor IL-15, as long as other cells, such as dendritic cells, express IL-15Rα and trans-present IL-15. Therefore, as IL-15 stimulates NK cells in the context of an immunological synapse (**Figure 1**), it has the potential of being subject to control by other receptor--ligand interactions, including signals from inhibitory receptors.

Stimulation of NK cells by type I IFN has interesting properties. Typically, type I IFN receptors signal through Stat1 and Stat2 to induce a cytolytic program. However, NK cells express a high basal amount of Stat4 that signals for IFN-γ secretion in response to type I IFN (131). Thus NK cells are “prewired” to produce IFN-γ before gradually switching from Stat4 to a Stat1-dependent expression of genes required for cytolytic function (131).

Sphingosine-1-phosphate (S1P) is an important mediator of lymphocyte trafficking out of lymphoid tissues. S1P binds to the three Gαi-coupled receptors S1PR1, S1PR3 and S1PR5 (132). T cell egress from lymph nodes is controlled by S1P binding to S1PR1, which is subject to desensitization through internalization after ligand binding. NK cells rely on a high expression of S1PR5, which is not subject to ligand-dependent desensitization, to detect S1P. S1PR5 is essential for NK cell migration; they accumulate in the bone marrow and lymph nodes in its absence (133, 134). S1PR5 is also resistant to downregulation mediated by CD69 (132). Thus, NK cells use a dedicated S1P receptor that promotes more rapid and efficient emigration from lymphoid tissue than that of T cells.

Signaling Requirements for NK Cell Priming, and for Innate and Adaptive Memory

NK cells in mice kept in pathogen-free conditions require "priming" in order to mount functional responses to viruses and parasites. Priming for responses to viruses depend on IL-15 trans-presentation by plasmacytoid DC that receive signals from type I interferon (135). Priming for NK responses to the parasite *Leishmania* requires IL-12 production by myleoid DCs (136). A proportion of NK cells isolated from human blood behave as primed cells, presumably due to priming by exposure to environmental microbes. NK cells in mice captured in the wild are also primed (137). The signaling requirements for priming and the exact state that defines NK cells as primed are not clear. Identification of markers or a transcriptional signature would facilitate their analysis.

NK cells can display memory-like functions after ex vivo stimulation with cytokines and transfer into naive mice (138, 139). This form of "conditioning" results in enhanced IFN-γ production but not cytotoxicity after restimulation. The changes in signaling that underlie this property have not been defined yet. Mouse NK cells exhibit innate memory properties after infection with MCMV and vaccinia virus (140, 141). Ly49H$^+$ NK cells in C57BL/6 mice are activated by the MCMV-encoded protein m157 expressed on infected cells (35, 37). Ly49H signals through the ITAM-bearing protein DAP12. The generation of long-lived Ly49H$^+$ NK cells with enhanced recall response to MCMV is dependent on IL-12 but not IFN-γ (142, 143). Finally, NK cells are also capable of mounting RAG-independent and yet adaptive memory-like responses to specific haptens and viral particles (144-146). Transfer of such memory NK cells into naive mice confers virus-specific protection from lethal challenge. Persistence of memory requires CXCR6, which is expressed on hepatic NK cells. The molecular basis for this type antigen-specific recognition and memory is still unknown.

Soluble HLA-G

All NK cells express CD158d (KIR2DL4), an unusual activation receptor that resides primarily in endosomes (33). Endosomal signaling by CD158d in response to soluble HLA-G results in a unique proinflammatory and proangiogenic response in the absence of cytotoxicity (147). Such a response would be relevant at sites of HLA-G expression, such as at the maternal-fetal interface during early pregnancy, which is characterized by an abundance of NK cells in the uterus, invasion of fetal trophoblast cells, and extensive vascular remodeling (13). Soluble HLA-G added to primary NK cells, freshly isolated from human peripheral blood, accumulates in the same endosomes where CD158d resides (147). Signaling from endosomes has recently emerged as a mechanism by which selected receptors provide sustained signals that are distinct from those generated at the cell surface. The signaling pathway triggered by CD158d in endosomes is distinct from the pathways utilized by endosomal TLRs and internalized growth factor receptors (33). The kinase activity of DNA-PKcs, a DNA damage signaling kinase, is required for CD158d signaling and phosphorylates Akt at serine 473. A CD158d--DNA-PKcs--Akt signaling axis culminates in NF-κB activation to generate proinflammatory and proangiogenic mediators (148). This novel mode of endosomal signaling could initiate sustained responses with particular relevance to vascular remodeling in response to fetal HLA-G during early pregnancy. Thus, interactions between an NK activation receptor and its soluble ligand can induce NK cell secretory responses that would favor implantation and reproductive success.

DOMINANT INHIBITION

To maintain equilibrium in biological systems, every biochemical signal must be calibrated by positive and negative control mechanisms. In addition to signal strength, the duration and oscillation of receptor signaling are often critical parameters that determine biological outcomes, and must be tightly regulated. A specific type of negative regulation, distinct

from negative feedback, occurs through dedicated inhibitory receptors that block activation signals at an early step, thereby preventing rather than terminating the intended outcome of activation (149). Such inhibitory receptors, which carry ITIM sequences in their cytoplasmic tail, are members of several receptor families and are expressed in many cell types (150, 151).

The concept and existence of the ITIM was first established with FcγRIIb, which inhibits BCR signaling by recruiting the inositol phosphatase SHIP when coligated through binding of IgG immune complexes (150, 152). However, the majority of ITIM-bearing receptors bind the tyrosine phosphatases SHP-1 and SHP-2. The precise ITIM sequence that provides specific binding sites for SHP-1 is V/IxYxxL/V (in single-letter amino acid code, x indicating nonconserved positions, and V/I indicating either valine or leucine), as determined with the first functional characterization of inhibitory KIR (153). Two ITIMs, separated by ~25 amino acids, in the cytoplasmic tails of inhibitory receptors provide a specific binding site for the tandem SH2 domains of SHP-1 or SHP-2 (153-155). The N-terminal cytoplasmic tail of the lectin-like Ly49 inhibitory receptors for MHC-I in the mouse carries a single ITIM. But as a covalent homodimer, each Ly49 receptor complex has two ITIMs. In contrast to inhibitory signaling by FcγRIIb, inhibition by KIR requires recruitment of SHP-1 or SHP-2, but not SHIP (153, 156).

The identification of the ITIM sequence led to the correct prediction that other ITIM-bearing receptors, including molecules of unknown function such as NKG2A, would have inhibitory functions. ITIM-bearing receptors have turned out to be widespread, and to regulate many cellular functions in different types of cells (150, 151, 157). In the case of NK cells, MHC-I specific ITIM-bearing receptors have a prominent role in controlling their cellular reactivity. Their characterization has led to the delineation of a conserved ITIM sequence and the discovery of many other receptors with similar inhibitory signaling properties.

ITIM-Bearing Inhibitory Receptors

As ITIM-based inhibition is dominant over activation signals received from receptors bound to ligands displayed on target cells, NK cell responses are not the outcome of a simple balance of signals for activation and inhibition. Rather, they involve a complex integration and hierarchy of signals. Recruitment of SHP-1 by MHC-I--specific ITIM-bearing receptors blocks signaling at a proximal step, such that most downstream signals are prevented from occurring at all, as opposed to being switched off (151). Thus, absence of a tyrosine-phosphorylated protein during inhibition, which would normally be phosphorylated during NK cell activation, should not be interpreted as being the result of SHP-1--mediated dephosphorylation. The only SHP-1 substrate to have been identified directly during inhibition of NK cells is Vav1, using a KIR--SHP-1 fusion protein that included a “substrate trapping” mutation in SHP-1 (158, 159). These experiments demonstrated that inhibition of NK cell lines by KIR--SHP-1 bound to HLA-C on target cells was mainly through dephosphorylation of Vav1. These and other data led to a new model for inhibition: ITIM-bearing receptors block NK cell activation upstream of actin-dependent signals, including signals required for the recruitment and phosphorylation of activation receptors (151). In support of this model, live imaging of nascent inhibitory receptor microclusters, stimulated synchronously by photoactivation of an HLA-C–peptide complex, showed that they form within seconds and immediately suppress the formation of activating receptor microclusters (160). This induced inhibition resulted in collapse of the peripheral F-actin network and retraction of the NK cell synaptic footprint (160).

What are the points at which activation signals are blocked by coengagement of inhibitory receptors? LFA-1--dependent adhesion of resting NK cells to target cells expressing ICAM-1 is enhanced by inside-out signals delivered by any one of several coactivation

receptors (17, 118). Signals as proximal as inside-out signals are blocked by coengagement of inhibitory receptor CD94--NKG2A by HLA-E on target cells (17). Outside-in signals delivered by LFA-1 are also blocked by inhibitory receptors (15). Natural cytotoxicity requires the combination of signals for granule polarization and for granule fusion with the plasma membrane. Experiments with reconstituted target cells expressing different ligands of NK activation receptors have shown that LFA-1--dependent and NKG2D--dependent granule polarization is more easily inhibited than degranulation induced by ADCC (Table 1) (94). However, interaction of NK cells with human target cells expressing MHC-I ligands of inhibitory receptors results in complete inhibition of polarization and degranulation (94). This suggests that inhibition may be facilitated by other receptor--ligand interactions.

Inhibitory signals block activation at a very proximal point, upstream of inside-out signals to, and outside-in signals by LFA-1, and upstream of actin-dependent signals for receptor recruitment and phosphorylation (151, 160). This may explain how inhibition dominates over most activation signals. Rather than turning activation signals off, inhibitory receptors prevent activation signals from occurring in the first place. An intriguing exception to the dominance of inhibition over activation---other than during activation by soluble molecules---may occur with the chemokine receptor for fractalkine (CX3CL1), a chemokine that exists as a transmembrane protein on DC. Not only does CX3CR1 transmit signals in the presence of coengaged inhibitory receptors, but it also overcomes the inhibition of other NK activation signals (161). The basis for this unusual property is not known.

The consequence of inhibitory receptor engagement may differ among effector and memory T cells. KIR^+ CTL clones isolated from tumor-infiltrating lymphocytes were inhibited by HLA-C ligand on tumor target cells (162). Early signals, such as phosphorylation of ZAP-70 and Vav1, TCR accumulation at the synapse, and actin rearrangement were all blocked, similar to inhibition of NK cells by $HLA\text{-}C^+$ target cells. In contrast, in KIR^+ memory T cells stimulated with superantigen, engagement of inhibitory KIR by HLA-C did not block adhesion, early Ca^{2+} signals and degranulation (163). Inhibitory receptor movement to the center of the synapse was delayed by 15 to 30 minutes and only later signals, including the transcriptional response were inhibited (163). Thus, the outcome of inhibitory KIR engagement on memory T cells is very different from the inhibition of proximal signals by KIR in NK cells.

Another property of inhibitory KIR that may be unique to T cells was observed after transfection of KIR into $CD4^+$ T cells. Surprisingly, $CD4^+$ T cells expressing KIR in the absence of its HLA-C ligand mounted stronger TCR-dependent IL-2 responses (164). Even in the absence of ligand, the KIR ITIM sequences were phosphorylated and bound to SHP-2. Immunological synapses formed in the presence of an HLA-C ligand accumulated phosphorylated KIR, recruited SHP-1, and blocked IL-2 production. The basis for the costimulatory property of a functional inhibitory KIR that is expressed on T cells, but not ligated, is still unknown. There is undoubtedly much more to discover about the properties of inhibitory receptors for MHC-I on non-NK cells, and about the specific signaling properties of the many other ITIM-bearing receptors.

A Signal Transmitted Directly by Inhibitory Receptors

Inhibitory receptors do not block NK cells on their own, in the absence of activation signals, but they have the capacity to block activation signals delivered by different types of activation receptors. Inhibition is effective only locally; it blocks signaling by coclustered activation receptors and does not impair NK cell responses to other stimuli (165, 166). This led to the appropriate term “coinhibition” to describe the properties of ITIM-bearing receptors (167).

Recent work has revealed that ITIM-bearing receptors can, in fact, signal independently. During inhibition of NK cells by MHC-I expressed on target cells, the small adaptor molecule Crk becomes phosphorylated and associates with the tyrosine kinase c-Abl (159). Crk links the GEF C3G, which activates the GTPase Rap1, with scaffold proteins in the cytoskeleton (168). As a result of phosphorylation, Crk dissociates from c-Cbl--Crk--C3G and $p130^{CAS}$--Crk--C3G complexes (168). Crk phosphorylation may contribute to inhibition of NK cells (159). HLA-E is sufficient to induce Crk phosphorylation in $NKG2A^+$ NK cells, as shown by imaging NK cells over lipid bilayers carrying HLA-E only (169). Crk is required for signaling by CD16 and for the movement of microclusters of CD16 ligands on the lipid bilayer (169). These data provide evidence that inhibition by CD94-NKG2A is achieved through two pathways, one leading to Vav1 dephosphorylation by SHP-1, the other to Crk phosphorylation by c-Abl (**Figure 3**). Thus, although ITIM-bearing KIR and CD94-NKG2A remain coinhibitory receptors, they have an additional property, namely that of signaling autonomously. It is still unknown how c-Abl is recruited and activated to phosphorylate Crk in the context of NK cell inhibition.

A combination of quantitative proteomics and modeling will be required to test how phosphorylation of Crk is achieved and the impact it has on the signaling network of NK cells during activation and inhibition. Modeling has been applied to investigate the mechanism by which ITIM-mediated inhibition is achieved (170-172). It will also be of interest to examine whether the many other ITIM-bearing receptors share the ability to activate c-Abl, and how this property may affect various cellular functions.

Contribution of HLA-Bound Peptides to Inhibition

Inhibition resulting from KIR recognition of MHC-I is influenced by the amino acid sequence of the peptide bound to MHC-I (173-176). While a wide array of peptides is competent to induce inhibition, recognition by KIR can be impaired by certain incompatible side chains at positions P7 and P8 of a nonamer peptide. The Ly49 inhibitory receptor counterparts in mice show no peptide preference, since the binding site of Ly49 on MHC-I is away from the peptide binding site (177, 178).

The peptide selectivity exhibited by inhibitory KIRs may have functional relevance. KIR-mediated inhibition of NK cell function can be relieved by HLA-C binding peptides that function as antagonists and interfere dominantly with inhibition mediated by peptides that strongly support KIR recognition of MHC-I (179). The molecular basis of this antagonism, which results in NK cell activation, is unknown. Interaction of inhibitory KIR with an antagonist peptide-loaded HLA-C may interfere with KIR bound to agonist peptide-loaded HLA-C, at the level of receptor clustering or inhibitory signaling, or it may deliver a signal that counteracts the typical ITIM-based inhibitory signal (179, 180). Regardless of the mechanism, peptide antagonism may allow NK cells to exploit the peptide selectivity of their inhibitory receptors to sense alterations in their environment and favor host responses to pathogens. In this way, NK cells can potentially respond rapidly to changes in peptide repertoire, both self-peptides and virus or tumor derived peptides generated during viral infections or in cancer. However, due to the extensive polymorphism of HLA ligands for the germ-line encoded KIR, NK cells cannot discriminate between self and nonself peptides. But this unexpected peptide antagonism, which overrides inhibition, could favor the host if new peptides bound to HLA-C during virus infection include antagonist peptides. It will be interesting to investigate if such a mechanism is what led to the KIR-associated selection of HIV variants in chronically infected individuals, similar to the selection of mutations in epitopes of virus-specific T cells (181, 182).

INHIBITORY RECEPTORS DETERMINE NK CELL RESPONSIVENESS

The original concept of "missing self" (183) was challenged when the first β_2-microglobulin deficient mice were generated. Despite the near absence of cell surface MHC-I in those mice, NK cells still exist, appear normal, but do not kill autologous MHC-I deficient cells (184, 185). The killing of MHC-I negative cells by wild-type NK cells had led to the missing self hypothesis, but a new concept was required to explain the self-tolerance of NK cells in an environment devoid of MHC-I. Recent findings have shown that NK cells adapt to the MHC-I environment, and are hyporesponsive in the absence of MHC-I or in the absence of inhibitory receptor on NK cells (5, 186). This adaptation may be similar to that of chronically stimulated lymphocytes, except that NK cells are stimulated mainly in the absence of MHC-I. The "Adaptable Lymphocyte Hypothesis" of Grossman and Paul states that "immune responses are elicited by fast increases in antigen […] and are both transient and aggressive" (187). In contrast, sustained or frequent exposure to antigen raises the activation threshold of lymphocytes. Their "Tunable Activation Threshold" model (187) can be applied to explain the adaptation of NK cells to the absence of MHC-I, which leads to sustained activation, and how NK cells detect *loss* of MHC-I but not the *absence* of MHC-I (**Figure 4**). A similar model, called "disarming", has been proposed to explain how NK cells devoid of an inhibitory receptor for self MHC are unresponsive, even in the presence of MHC-I (**Figure 4**) (186, 188).

NK Cells Adapt to the MHC-I Environment

The intrinsic responsiveness of NK cells to activation stimuli is determined by the interaction of inhibitory receptors with MHC-I in a way that is dependent on the ITIM (5). NK cells that lack ITIM-bearing inhibitory receptors for self MHC-I and NK cells from hosts that lack MHC-I ligands for ITIM-bearing inhibitory receptors have a reduced responsiveness to activation signals, such as crosslinking of an activation receptor or stimulation by sensitive target cells (5, 186, 189, 190). These results led to two models. The first—disarming—proposes that in the absence of inhibition, continuous stimulation of NK cells leads to a state of unresponsiveness, also referred to as hyporesponsiveness (188). The disarming model is simple, as it requires only that inhibitory receptors do their job, namely inhibit NK cells in the presence of MHC-I ligands. The second model proposes that inhibitory receptors provide an ITIM-dependent instructive signal to the NK cells that renders them responsive (191). The term "licensing" used to describe the property of inhibitory receptors that renders NK cells responsive (5) was initially understood as representing the second model, also known as "arming" (188, 192). Licensing is now understood to encompass any process by which NK cells that receive signals through inhibitory receptors for MHC-I become responsive (193). The hyporesponsiveness of NK cells due to the lack of ITIM-dependent signaling can be partially overcome by cytokines (5). Therefore, the lack of licensing may not be a handicap for NK cell responses during inflammation, as shown in the response to MCMV infection (194, 195).

It is important to note that the arming and disarming models are not mutually exclusive. Evidence for disarming comes from early experiments in vitro, with transgenic mice that have "mosaic" expression of MHC-I, and using cell transfers into mice (196, 197). If NK cells exposed to an environment with mixed MHC-I positive and negative cells are not rejecting MHC-I deficient cells, arming cannot be the sole or dominant mechanism to develop responsiveness (192). Also consistent with disarming, NK cell transfers into transgenic mice that express m157, an MCMV-encoded ligand for activation receptor Ly49H, resulted in reduced NK cell responsiveness, despite normal expression of MHC-I and of inhibitory receptors (198, 199). NK cells may even be subject to disarming in wild-type mice. A defective mutant of activation receptor NKp46 results in NK cell hyperactivity,

suggesting that signaling by wild-type NKp46 may partially disarm NK cells (200). Downregulation of NK cell responsiveness in the presence of MHC-I could be explained if a stronger inhibition is required to promote arming than that to block responses of licensed NK cells. Indeed, licensing requires stronger inhibitory signals than MHC-I dependent inhibition of target cell killing, consistent with an arming mechanism (201).

Conversely, responsiveness can be restored to unresponsive NK cells by transfer from MHC-I deficient mice into MHC-I^{+} hosts. Responsiveness is acquired independently of maturation, cell division, and of MHC-I expression by NK cells, consistent with either arming or absence of disarming (202, 203).

The arming model proposes that licensing is achieved by an ITIM-dependent signal that is distinct from the dephosphorylation by tyrosine phosphatases SHP-1 or SHP-2 of substrates such as Vav1 (191, 192). ITIM-bearing KIR and CD94-NKG2A are capable of delivering such a signal. During inhibition of NK cells by an inhibitory receptor bound to MHC-I on target cells, the small adaptor molecule Crk becomes phosphorylated, associates with the kinase c-Abl, and dissociates from signaling complexes (159). Furthermore, CD94-NKG2A can deliver this signal autonomously, as binding to HLA-E attached to artificial lipid bilayers is sufficient to induce phosphorylation of Crk (169).

Irrespective of the mechanism(s) for licensing, NK cell responsiveness is calibrated by a dynamic process according to the strength of inhibitory signals received (190, 204, 205). A “rheostat” model has been proposed to account for the quantitative tuning of NK cell responsiveness (192, 205, 206). NK cells adapt continuously to the MHC-I environment and are activated to kill only by the sudden loss of MHC-I on target cells, as may occur during viral infections or cell transformation (**Figure 4**).

A Revocable License

What is the molecular basis of licensing (also referred to as education or tuning), and how different are licensed and unlicensed NK cells? The transcriptional signatures in three different sets of licensed and unlicensed NK cells have revealed very little difference (207). One comparison was between NK cells in wild-type and β_2-microglobulin deficient mice; the other between wild-type and mice lacking the H-2D and H-2K MHC-I genes; and, third, MHC-I-negative mice in which NK licensing was provided by transgenic coexpression of human HLA-Cw3 and KIR2DL3 (207). Although 45 to 430 genes were differentially regulated within each set, only one gene was upregulated in licensed NK cells among all three independent sets, namely Klra6 (Ly49F), coding for an inhibitory isoform of Ly49 (207). If NK cell licensing were to be determined by a selection process during development, a distinct cell fate, a functional maturation step, or by something similar to T cell anergy, a transcriptional signature should have been apparent. Therefore, it is more likely that the tuning of NK cell responsiveness is set by transient signals, which could also explain the reversibility of licensing. Whether changes in the phosphorylation status of Crk mediated by ITIM-containing receptors could account for licensing is not known, but they do suggest an updated model for licensing, referred to as the revocable license.

It is important to note that licensing by inhibitory receptors renders NK cells more responsive to subsequent activation signals that are delivered in the absence of inhibition. It does not render NK cells resistant to inhibition. One of the properties of licensed mouse NK cells is a reduced confinement of activation receptors NK1.1 and NKp46 at the plasma membrane, as measured by dynamic fluorescence correlation spectroscopy (207). Unlicensed, hyporesponsive NK cells display actin-dependent confinement of these receptors. Although counterintuitive, the phosphorylation of Crk could contribute to both inhibition and licensing. As Crk is required for signaling by activation receptors (169), its

inactivation by phosphorylation is an efficient way to block NK cell activation. At the same time, phosphorylation of Crk and its dissociation from cytoskeletal complexes would disrupt the F-actin network and reduce the constraint imposed by F-actin on receptor movement. Upon subsequent activation, and due to their greater lateral diffusion, activation receptors would have an enhanced ability to form signaling microclusters. Shortly thereafter, in the absence of inhibition, dephosphorylated Crk will reassemble into signaling complexes, the F-actin network will be restored, cytoskeletal constraints will confine activation receptors, and the activated NK cells will lose their license for future responses. This revocable license model could also account for the rapid and reversible tuning of NK cell responsiveness. NK cells would keep their license as long as they remain inhibited and under control. Once they lose inhibitory control through activation by MHC-I negative cells their license would be revoked.

CONCLUDING REMARKS

A better understanding of how NK cell responses are controlled through simultaneous engagement of several germ-line encoded receptors has begun to emerge and has led to the formulation of a few basic principles. Multiple checkpoints are in place to avoid uncontrolled cytotoxicity and proinflammatory responses. Pairs of coactivation receptors have to synergize to overcome negative regulation by a ubiquitin ligase. The polarization of lytic granules to the cytotoxic synapse and the fusion of granules with the plasma membrane are under independent control of complementary signals. Inhibitory receptors specific for MHC class I molecules had been considered coinhibitory, given their dedicated role in blocking activation signals. Through their inhibitory function, these receptors contribute also to the maintenance of the intrinsic responsiveness of NK cells. In addition, these ITIM-bearing receptors have the capacity to deliver an autonomous signal for the phosphorylation of an adaptor molecule. Their signaling potential is thus greater than initially thought, and suggests a new model for the rapid tuning of NK cell responsiveness. NK cells have again contributed to a deeper understanding of fundamental cellular processes and their regulation, which is relevant to the ITIM-dependent control of many types of cellular responses in various cell types. We end with a list of issues worthy of further investigation.

FUTURE ISSUES

- What are the signaling properties of the different activating receptors on NK cells and how are these signals integrated spatially and temporally?
- How does the integrin LFA-1 signal for the polarization of lytic granules?
- How do signals from multiple receptors combine for the detection of tumor cells, infected cells, and APC and activated T cells during immune responses?
- What is the contribution of non-MHC-specific inhibitory receptors to NK function? How is the expression of adaptor SAP regulated to balance the activating and inhibitory properties of the SLAM family of receptors?
- What is the signaling basis for licensing of NK cells? Are there distinct ITIM-dependent pathways that lead to inhibition or licensing?
- What is the basis for the genetic associations that implicate inhibitory receptors and their MHC-I ligands in disease outcome? Are they due to inhibition of NK cell responses or enhanced licensing by inhibitory receptors to promote greater NK responsiveness?

- What signals are involved in peptide antagonism of inhibitory KIR signaling to prevent NK cell inhibition? How do virus infections change the balance of agonist and antagonist peptides?
- What molecular events control the induction and maintenance of NK cell priming and NK cell memory?
- Is there a new genetic system that generates antigen receptor diversity for NK adaptive immunity to specific haptens and viruses?

COMMON MISCONCEPTIONS

1. The "major NK activating receptors" NKG2D and NKp46 are not sufficient on their own to activate degranulation by primary resting NK cells. Unlike T cells, NK cells do not have a single activation receptor, but rather coactivation receptors that synergize to activate NK cells. Receptors previously considered "costimulatory," such as 2B4 and DNAM-1, are also coactivation receptors.
2. "Paired" sets of activating and inhibitory receptors are not linked functionally, with the ITIM serving as inhibitor of the "paired" ITAM. ITIM-based inhibition is independent of ITAMs and blocks many types of activation signals.
3. There is no "peptide specificity" in the recognition of HLA by KIR, but only peptide selectivity based on discrimination against unfavorable peptide sequences. Moreover, due to HLA polymorphism and the germ-line encoded KIR repertoire, NK cells cannot discriminate between self and viral peptides.
4. $CD56^{dim}$ NK cells are not "poor cytokine producers." Capable of robust secretory responses, they respond better to cell surface ligands, while $CD56^{bright}$ cells respond better to soluble factors.
5. KIR2DL4 is not an inhibitory receptor and primary NK cells are not globally inhibited by HLA-G. KIR2DL4 is an activation receptor for soluble HLA-G and induces a secretory response in all NK cells.

Acknowledgments

We thank A. Hoofring for illustrations and L. M. Thomas for comments. This work was supported by the Intramural Research Program of the National Institutes of Health, National Institute of Allergy and Infectious Diseases.

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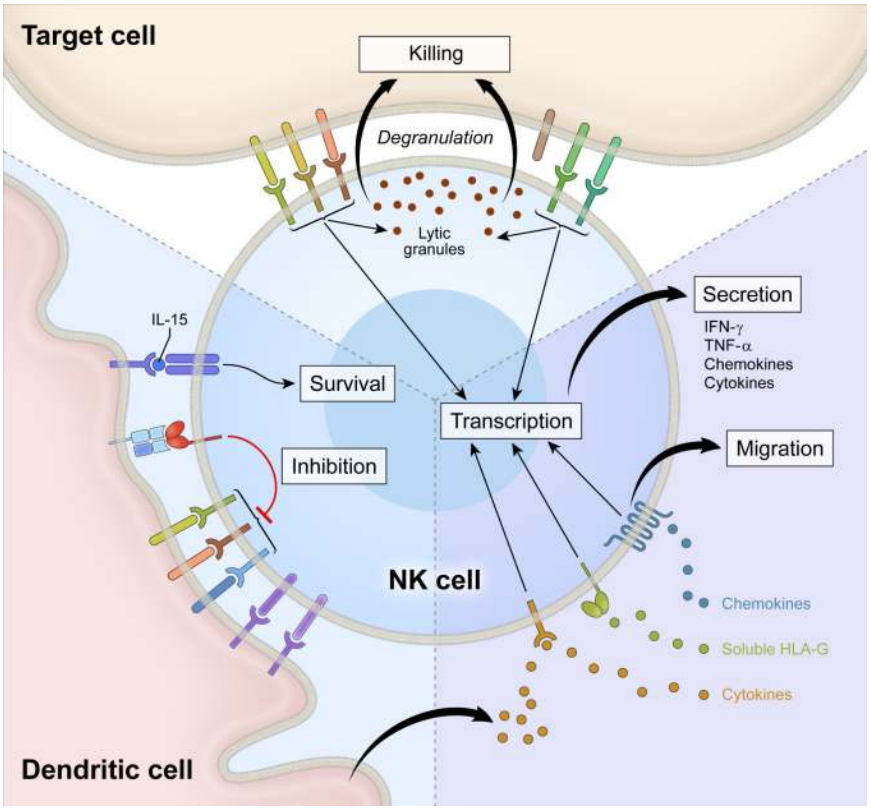

Figure 1.

Multiple signals control NK cell responses. An array of NK cell activation receptors binds to ligands on other cells. Target cell killing requires signals for lytic granule polarization and degranulation. Combinations of coactivation receptors synergize to induce killing and a secretory response. Some of the secretion response is independent of transcription. Inhibitory receptors (*Red*) for MHC-I exert dominant inhibition of cytotoxicity and secretion, as seen in the NK--DC interaction. DC also receive signals from ligands on NK cells. In the absence of inhibition through MHC-I, target cells that express ligands for activation receptors are killed. NK cells respond to various soluble activators such as cytokines and chemokines that deliver signals for functions such as migration and survival. The prosurvival cytokine IL-15 bound to the IL-15R α chain is trans-presented by other cells to the IL-15R $\beta\gamma_c$ chains on NK cells. In response to soluble factors, NK cells secrete IFN-γ, TNF-α, other cytokines, and chemokines. Signals received through receptors that bind soluble ligands are not subject to inhibition through MHC-I. Soluble HLA-G binds to CD158d and induces a transcriptional response for a unique set of proinflammatory, proangiogenic molecules.

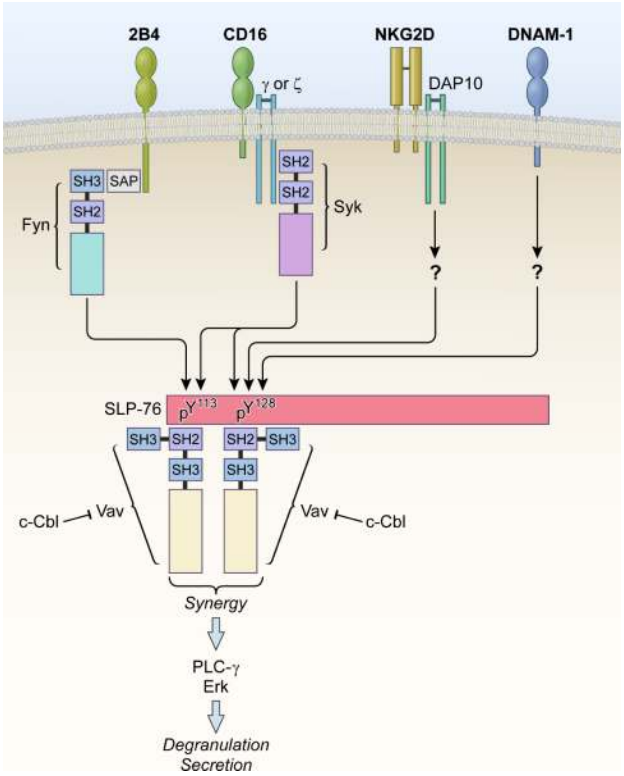

Figure 2.

A molecular basis for synergistic activation through pairs of coactivation receptors on NK cells. Cytotoxicity and secretion by primary resting NK cells requires synergistic signals from two coactivation receptors, such as 2B4 and NKG2D or 2B4 and DNAM-1. This synergy is required to overcome inhibition by the ubiquitin ligase c-Cbl of a Vav1-dependent activation pathway. Stimulation through 2B4 results in a Fyn-dependent, Syk-independent, selective phosphorylation of Tyr113, but not Tyr128 in the adaptor protein SLP-76. Conversely, stimulation through NKG2D or DNAM-1 results in selective, Syk-independent phosphorylation of Tyr128, but not Tyr113 in SLP-76. In contrast, stimulation by the Fcγ receptor CD16 results in Syk-dependent phosphorylation of both Tyr113 and Tyr128, as is the case after TCR signaling in T cells.

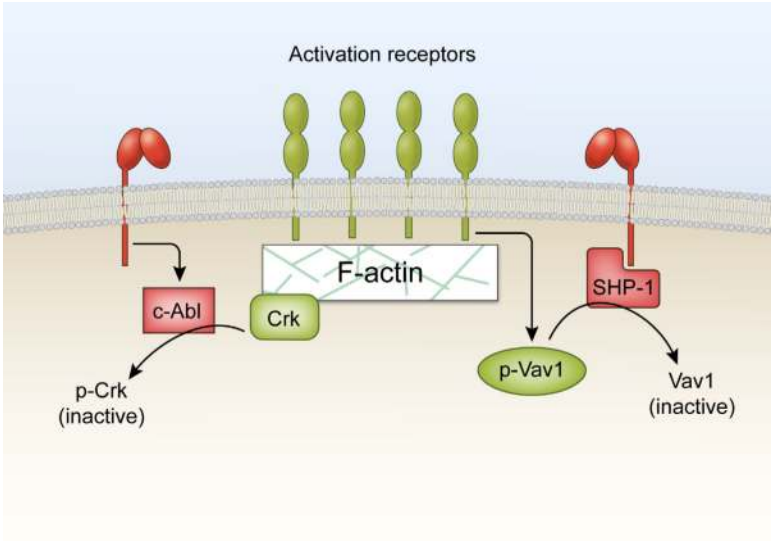

Figure 3.

Two components of negative signaling by inhibitory receptors for MHC-I. Signaling by activation receptors (*Green*) requires the adaptor molecule Crk, actin polymerization, and phosphorylation of the guanine exchange factor Vav1. Recruitment of tyrosine phosphatase SHP-1 by phosphorylated ITIMs in the cytoplasmic tail of inhibitory receptors (*Red*) results in Vav1 dephosphorylation. In addition, inhibitory receptors induce Crk phosphorylation by the tyrosine kinase c-Abl. Phosphorylated Crk dissociates from cytoskeletal signaling complexes that include the scaffold proteins c-Cbl, paxillin, and p130CAS.

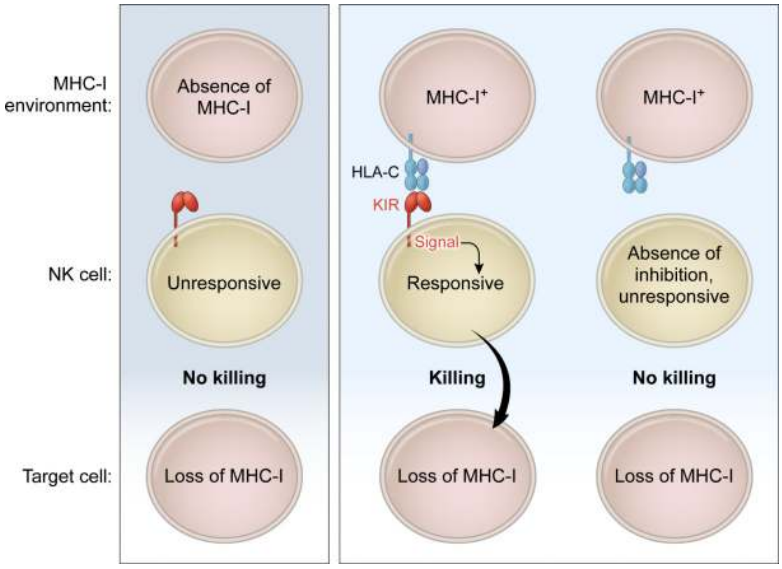

Figure 4.

NK cells detect the loss but not the absence of MHC-I. In the absence of self MHC-I, inhibitory receptors on NK cells are not engaged and NK cells remain unresponsive. In the presence of MHC-I, inhibitory receptors such as KIR deliver an inhibitory signal that prevents disarming and/or a signal that renders NK cells responsive (i.e., arming signal). The two options are not mutually exclusive and have the same outcome: NK cells become licensed by either maintaining or acquiring responsiveness, and they can kill target cells. NK cells that lack inhibitory receptors for self MHC-I are not licensed. Licensing is a calibrated process that provides quantitative tuning of NK cell responsiveness. For clarity, receptor--ligand combinations for activation are not shown.

Table 1

Activation and inhibition of primary NK cell responses by ligands on insect S2 cells

Receptor (Ligand)	Degranulation		Polarization	Killing	(References)
NKG2D *(ULBP1)*	No		No	No	(17, 87)
2B4 *(CD48)*	No		No	No	(17, 87)
NKG2D + 2B4	Yes[a]		No	No	(17, 87)
LFA-1 *(ICAM-1)*	No		Yes[b]	No	(15, 16, 94, 123)
NKG2D + 2B4 + LFA-1	Yes		Yes	Yes	(17)
CD16 *(anti-S2 IgG)*	Yes		No	No	(16, 17)
CD16 + LFA-1	Yes		Yes	Yes	(16, 94)
NKG2D + 2B4 + IR[c] *(HLA-E)*	No[a]		No	No	(17, 87)
LFA-1 + IR *(HLA-C, HLA-E)*	No		No	No	(94)
CD16 + IR *(HLA-E)*	Less		No	No	(17)
CD16 + LFA-1 + IR *(HLA-C, HLA-E)*	Yes	Less[d]	No	No	(94)

[a] Green shading indicates activation, red shading indicates inhibition of that signal.

[b] In mouse NK cells, polarization requires coengagement of LFA-1 with NKG2D (93).

[c] Inhibitory receptor (KIR and/or CD94-NKG2A).

[d] Tested with NK clones. Some clones were partially inhibited.