

Strategies of Natural Killer Cell Recognition and Signaling

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Abstract The participation of natural killer (NK) cells in multiple aspects of innate and adaptive immune responses is supported by the wide array of stimulatory and inhibitory receptors they bear. Here we review the receptor-ligand interactions and subsequent signaling events that culminate in NK effector responses. Whereas some receptor-ligand interactions result in activation of both NK cytotoxicity and cytokine

production, others have more subtle effects, selectively activating only one pathway or having distinct context-dependent effects. Recent approaches offer ways to unravel how the integration of complex signaling networks directs the NK response.

1

Introduction

Natural killer (NK) cells are large granular lymphocytes of the innate immune system. They are widespread throughout the body, being present in both lymphoid organs and nonlymphoid peripheral tissues (Cooper et al. 2004; Ferlazzo and Munz 2004). NK cells are involved in direct innate immune reactions against viruses, bacteria, parasites, and other triggers of pathology, such as malignant transformation, all of which cause stress in affected cells (Moretta et al. 2002; Raulet 2004). Importantly, NK cells also link the innate and adaptive immune responses, contributing to the initiation of adaptive immune responses (see chapter by Zitvogel et al., this volume) (Martin-Fontecha et al. 2004) and executing adaptive responses with the CD16 FcγRIIIA immunoglobulin Fc receptor. Such responses are mediated through two major effector functions, the direct cytotoxicity of target cells and the production of cytokines and chemokines. We focus here on the nature of recognition events by NK cells and address how these events are integrated to trigger these distinct and graded effector functions.

2

Themes of NK Cell Recognition

The dissection of NK cell innate recognition strategies was initiated by the discovery that NK cell cytotoxicity inversely correlates with the level of major histocompatibility complex (MHC) class I expression on target cells (Karre et al. 1986). The missing self hypothesis elegantly provided an explanation for this phenomenon and led to the discovery of multiple inhibitory receptors that block activating signals by recruitment of protein tyrosine phosphatases to their intracytoplasmic immunoreceptor tyrosine-based inhibition motifs (ITIMs) (Long 1999; Vivier and Daëron 1997). Opposing this inhibitory signaling, innate stimulatory recognition by NK cells can be classified in three general modes: recognition of constitutively expressed self molecules, recognition of motifs upregulated by stressed cells, and direct recognition of infectious pathogen components (Raulet 2004; Vivier and Malissen 2005). Together,

the numerous receptors carrying out these functions allow NK cells to discriminate between target and non-target cells (Fig. 1) (Cerwenka and Lanier 2001; Vilches and Parham 2002; Vivier and Biron 2002; Yokoyama 1998). Only a minority of these receptors, such as the natural cytotoxicity receptors (NCR), are truly NK cell specific, with many being found on other hematopoietic cells. The question of how all the signals are integrated from multiple, redundant and opposing, simultaneously engaged pathways to culminate in graded NK cell responses, that is, cytotoxicity and/or cytokine production, serves to illustrate the complex and dynamically balanced nature of cell activation (Lanier 2003; Vivier et al. 2004).

3

Inhibitory Recognition and Signaling

3.1

Inhibitory Receptors for MHC Class I

Multiple families of receptors in mouse and human recognize MHC class I products and transmit inhibitory signals when engaged. CD94/NKG2 receptors are heterodimers of C-type lectin type II transmembrane proteins that recognize the nonclassical MHC class I molecules HLA-E (human leukocyte antigen-E, in human) and Qa-1^b (in mouse) (Borrego et al. 1998; Braud et al. 1998; Lee et al. 1998; Vance et al. 1998). Of the NKG2 family members that associate with CD94 (including NKG2A, C, and E), NKG2A contains ITIMs in its cytoplasmic domain conferring inhibitory function. Recognition of HLA-E or Qa-1^b by CD94/NKG2 requires the presence of peptides in the peptide binding grooves of these class I molecules. Many of these peptides are derived from the leader sequences of classical MHC class I molecules (Braud et al. 1997; Kraft et al. 2000), thus making CD94/NKG2A a sensor of active MHC class I biosynthesis and presentation. CD94/NKG2 is unique in both its high evolutionary conservation and its means of recognizing classical MHC class I as a “proxy” sensor.

Members of other NK cell MHC class I receptor families directly bind to classical MHC class I molecules. In the mouse and rat, NK cell recognition of subsets of MHC class I allotypes is mediated by members of the Ly49 family of C-type lectin type II transmembrane proteins. In contrast, human NK cells use immunoglobulin domain-containing type I transmembrane proteins for the same function. The immunoglobulin-like transcript 2 (ILT2, or LIR1) receptor recognizes a broad range of both classical and nonclassical MHC class I molecules (Chapman et al. 1999; Colonna et al. 1997), whereas members

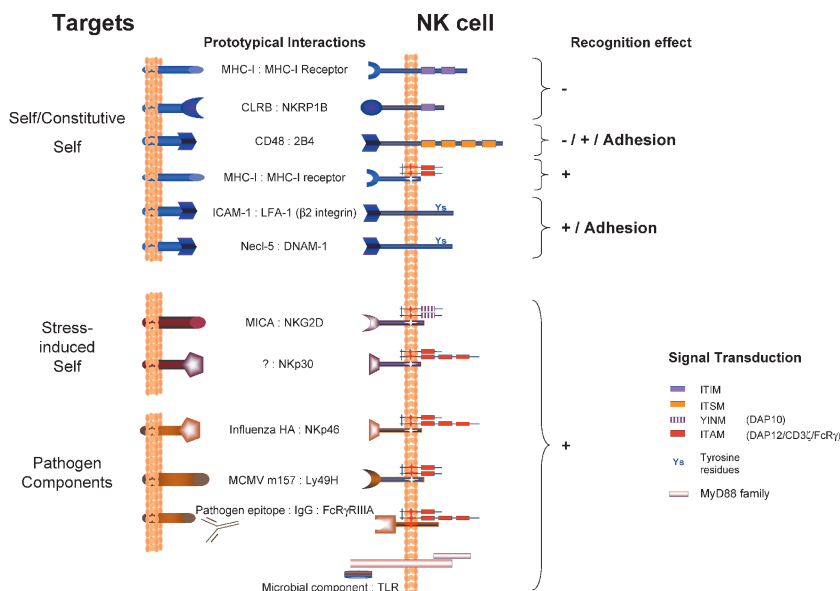


Fig. 1 Themes in NK cell recognition and signaling. NK receptors can be classified according to their recognition of ligands expressed by self cells, stressed cells, or pathogens. Prototypical receptor-ligand interactions that are described in the text are shown. Additional ligands are known for a number of the receptors shown. Effects of these interactions are detailed as inhibitory (–), activating (+), or involved in adhesion. The motifs used by membrane receptors or associated signaling adaptor molecules are shown for each receptor. These serve to illustrate the association of many NK activating receptors with specialized signaling transmembrane adaptor proteins that contain ITAMs (immunoreceptor tyrosine-based activation motifs) or YINM motifs in their cytoplasmic tails. These adaptors form homodimers or heterodimers through disulfide bonding and associate with membrane receptors using charged amino acids in the transmembrane domain (*stars*; *white* indicates positive charge, *red* indicates negative charge). Other motifs including ITIM (immunoreceptor tyrosine-based inhibitory motif) and ITSM (immunoreceptor tyrosine-based switch motif) are found within the cytoplasmic domains of NK receptors. The Fc γ RIIIA (CD16) receptor could be classified as recognizing constitutive self, stressed self, or pathogen-expressed moieties depending on a particular IgG antibody. As IgG antibodies recognizing constitutive self molecules are frequently avoided through processes of immune tolerance, Fc γ RIIIA is classified here as recognizing pathogen components. The figure includes both human and murine molecules that are not always found in the other species. Other important abbreviations: *MHC-I*, major histocompatibility complex class I molecule; *MICA*, MHC class I chain-related protein A; *Influenza HA*: influenza virus hemagglutinin; *MCMV*, murine cytomegalovirus; *TLR*, Toll-like receptor. Direct interaction and inhibitory modulation of NKp30 by pp65 of human cytomegalovirus has been described (Arnon et al. 2005). Therefore, NKp30 may also be defined as recognizing pathogen components

of the killer immunoglobulin-like receptor (KIR) family are much more specific. Primate KIR show MHC class I allotype specificity and appear to be functional homologs of rodent Ly49 despite their diverse evolutionary origin. Hitherto-studied mammalian species segregate into those with an expansion of *KIR* genes or *Ly49* genes (Parham 2005). An interesting feature of both KIR and Ly49 recognition of MHC class I is sensitivity to peptides bound in the MHC class I groove. Many KIR and some Ly49 receptors are sensitive to peptide changes (Franksson et al. 1999; Hanke et al. 1999; Hansasuta et al. 2004; Peruzzi et al. 1996; Rajagopalan and Long 1997; Zappacosta et al. 1997), but this sensitivity is far less pronounced and specific than that underpinning T cell antigen recognition. The different roles of peptide are also reflected in the binding kinetics of NK and T cell MHC class I receptors. Although both types of receptor have similar affinities, KIRs have fast on and off rates with favorable binding entropy whereas T cell receptors (TCR) have slower kinetics (Maenaka et al. 1999; Vales-Gomez et al. 1998). The slow on rate of the TCR is relatively peptide independent and may reflect an energetically unfavorable transition state in which peptide-independent TCR-MHC contacts are made. Peptide has a large influence on the off rate, however, reflecting its importance in complex stability and role in T cell signaling (Wu et al. 2002).

NK recognition of individual allotypes of MHC class I could play an important role in innate immunity against viruses or tumorigenic processes that result in the loss of expression of specific MHC class I allotypes (Garcia-Lora et al. 2003). In addition, the polymorphic nature of both NK receptors and their MHC ligands suggests that a population effect may be at work, with NK receptors serving to modify the NK cell activation state (see chapter by Carrington and Martin, this volume) (Parham 2005). This is illustrated in the finding that preeclampsia, a complication associated with insufficient remodeling of the uterine spiral arteries that provide a blood supply for the fetus during pregnancy, is associated with HLA-C group 2 molecules that could theoretically provide strong inhibitory signals to decidual NK cells (Hiby et al. 2004). It is also known that NK KIR-HLA interactions can be exploited during certain bone marrow transplantation procedures where incompatibility decreases the likelihood of graft-versus-host disease and leukemia relapse (Ruggeri et al. 2002). Of importance in each role is the repertoire of NK cell specificity. Almost all NK cell MHC class I receptors are expressed in a varied fashion by the NK population, resulting in a broad range of MHC class I specificities within any individual's NK population (see chapter by Anderson, this volume).

3.2

Inhibitory Receptors for Non-MHC Ligands

A number of NK receptors whose ligands are non-MHC class I molecules also contain ITIMs and have the capacity to function as inhibitory receptors. These include glycoprotein 49 B1 (gp49B1) and certain NK cell receptor protein 1 (NKR-P1) family members found only in mice, along with carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1) and sialic acid-binding immunoglobulin-like lectin (SIGLEC) family members found in both humans and mice. The roles of these receptors are currently obscure, but the broad expression of many of their ligands suggests that some may perform “missing-self” functions in a similar way to MHC class I receptors (see chapter by Plougastel and Yokoyama, this volume) (Kumar and McNerney 2005).

4

Activating Recognition and Signaling

4.1

Some ITAM-Based Receptors

In a similar way to the antigen receptors expressed by T and B cells, many of the NK receptors that induce strong activation of NK cells upon receptor cross-linking or ligation use immunoreceptor tyrosine-based activation motifs (ITAMs) to transduce these signals. In many cases these ITAMs are not present in the polypeptide conferring ligand binding capacity but are connected with it through noncovalent association of a transmembrane ITAM-bearing adaptor molecule. DNAX-activating protein of 12 kDa [DAP12, or KARAP (killer cell activating receptor-associated protein)], FcR γ , and CD3 ζ are such signaling adaptors expressed by NK cells and involved in signal transduction from multiple distinct surface receptors. Two members of the NCRs, NKp46 and NKp30, along with the Fc γ RIIIA CD16 receptor responsible for NK cell antibody-dependent cellular cytotoxicity (ADCC) couple with FcR γ and CD3 ζ . The other NCR, NKp44, couples with DAP12. As previously stated, NCRs are NK cell-specific receptors. Their name derives from their critical involvement in natural cytotoxicity against a broad panel of target cell types without prior NK cell sensitization, as demonstrated by antibody blocking of the cytotoxicity (see chapter by Bottino et al., this volume). Despite the reported NCR interaction with viral products (Arnon et al. 2005; Mandelboim et al. 2001), the identity of the NCR ligands expressed by tumor cells is still unknown, but it is tempting to speculate that these could either be normal self molecules or moieties upregulated on stress (Bloushtain et al. 2004).

4.2

NKG2D

NKG2D is an outlying member of the NKG2 family that forms homodimers and does not heterodimerize with CD94 (see chapters by González et al. and Jabri and Meresse, this volume). Many NKG2D ligands are class I MHC-related molecules that are expressed very selectively or at low levels by normal cells but are upregulated during stress and cellular transformation. In humans, NKG2D ligands include MICA, MICB, and various ULBP/RAET1 molecules, and murine H60, MULT1, and Rae1 molecules provide these roles in the mouse. The NKG2D ligands MICA, MICB, and Rae1 are all upregulated in tumor cells (Cerwenka et al. 2000; Diefenbach et al. 2000; Groh et al. 1996). NKG2D stimulation on NK cells leads to strong activation, and transfection-mediated NKG2D ligand expression on tumor cells leads to their rapid rejection in syngeneic mice (Cerwenka et al. 2001; Diefenbach et al. 2001). NKG2D is also expressed by CD8⁺ T cells. However, NKG2D stimulation is not sufficient to induce effector functions of CD8⁺ T cells without a primary activating signal coming from, for example, the TCR. The inability of NKG2D to directly stimulate CD8⁺ T cells without additional signals is partially a consequence of its method of signal transduction. Human NKG2D and the long splice-variant form of mouse NKG2D (NKG2D-L) associate exclusively with the DAP10 (DNAX-activating protein of 10 kDa) signaling adaptor. Unlike the signaling adaptors used by the NCRs, DAP10 contains a YxxM motif that links to signaling pathways distinct from those of ITAM-bearing receptors. In the mouse, an additional short splice-variant form of NKG2D (NKG2D-S) is able to associate with both DAP10 and DAP12, but the lack of DAP12 expression in the majority of T cells restricts its association to the NK cell compartment (Diefenbach et al. 2002; Gilfillan et al. 2002).

4.3

Activating Homologs of Inhibitory MHC Class I Receptors

All the families of MHC class I receptors previously mentioned in the context of their inhibitory function also include activating molecules. Activating members of the Ly49, KIR, and NKG2 families (excepting NKG2D, which does not complex with CD94) are highly homologous to inhibitory receptors within these families but contain no ITIM and associate with DAP12. Where it has been possible to show direct binding to MHC class I molecules, the affinities of these activating interactions are much lower than those of activating counterparts (Vales-Gomez et al. 2000), questioning whether MHC class I molecules are their functional ligands. For the activating Ly49s, an astonishing role for Ly49H has been demonstrated in the control of murine

cytomegalovirus (MCMV) infection. This receptor directly binds the MCMV *m157* gene product and is critical in control of infection by certain strains of mice (see chapters by Vidal and Lanier and Gumá et al., this volume). In addition, the activating receptor KIR2DS4 has been reported to bind a non-MHC class I ligand expressed by melanoma cells, suggesting a role in recognition of altered self (Katz et al. 2004).

It is not yet known whether such MHC class I-independent functions also apply to other activating members of these families. The high genetic polymorphism of activating receptors from the Ly49 and KIR families and a recent report supporting the continuous evolution of activating genes from inhibitory genes suggest that strong positive and negative evolutionary pressures are acting on them (Abi-Rached and Parham 2005). These pressures could be due to host-pathogen interactions in which pathogen “decoy” molecules for inhibitory receptors become detectable by a newly evolved activating receptor, giving a host with this receptor the upper hand (Arase and Lanier 2002). Alternatively, the selection pressures may be entirely due to interactions with self-MHC molecules. Support for this comes from disease association studies in which both KIR and HLA have been studied in parallel. The genetic combination of an activating KIR and its known or potential (based on inhibitory KIR homology) HLA ligand is beneficial during HIV or hepatitis C virus (HCV) infection but increases the risk of developing certain autoimmune diseases including type I diabetes and psoriasis vulgaris (see chapters by Carrington and Martin and Johansson et al., this volume). Activating KIRs are also reported as beneficial during pregnancy, where their genetic presence reduces the risk of preeclampsia (Hiby et al. 2004). These findings point to a MHC-based role for activating KIR in regulation of immune responses and/or homeostasis of the NK and T cells that bear them.

Unlike activating *KIR* and *Ly49*, the *NKG2C* and *E* genes are highly conserved, suggesting a long-running evolutionary pressure for their maintenance. Along with changes in CD94/NKG2C expression observed on NK and T cells during infection, this argues for a role in modifying the activation capacity of these lymphocytes (see chapter by Gumá et al.).

5

The NK Cell Activation Cascade: From Surface Triggers to Effector Function

Engagement of NK-activating receptors induces tyrosine phosphorylation of their associated adaptor proteins. After phosphorylation by Src family kinases, ITAM-containing adaptors recruit the protein tyrosine kinases Syk

and ZAP70, ultimately triggering NK cell effector functions. Alternatively, the YINM motif of DAP10, which signals for the receptor NKG2D (Wu et al. 1999), binds the p85 regulatory subunit of phosphatidylinositol 3-kinase (PI3K) (Chang et al. 1999; Wu et al. 1999) and the adaptor Grb2 (Chang et al. 1999) upon tyrosine phosphorylation.

Researchers are now actively exploring membrane-distal signaling molecules that mediate NK cell effector functions, particularly cytotoxicity. This process involves several steps including (a) rearrangement of actin cytoskeleton and formation of an immune synapse (IS) between NK cells and target cells, (b) reorientation of the Golgi complex and microtubule organizing center (MTOC) to polarize the lytic granules toward the IS, and (c) release of lytic granule contents (perforin and granzymes). The Vav guanine nucleotide exchange factors are implicated in all three of these processes as they play a central role in the activation of GTP-binding proteins (Billadeau et al. 1998; Chan et al. 2001; Colucci et al. 2001; Galandrin et al. 1999). Specifically, Vav1 is required for DAP10-mediated cytotoxicity, whereas Vav2 and Vav3 are essential for ITAM-mediated cytotoxicity (Cella et al. 2004). The Rho family of GTP-binding proteins (Rac1, RhoA, and Cdc42) and the Wiskott-Aldrich syndrome protein (WASP) regulate cytoskeleton rearrangements required for IS formation and MTOC-directed granule polarization (Gismondi et al. 2004; Khurana and Leibson 2003). Ras-related GTPase Arf6 promotes the release of cytolytic granule contents (Galandrin et al. 2005). Specifically, Arf6 activates the phosphatidylinositol 4-phosphate 5-kinase type I α (PI5KI α), contributing to the generation of a phosphatidylinositol 4,5-bisphosphate (PIP₂) plasma membrane pool required for granule secretion.

Exocytosis of lytic granules also involves phospholipases C γ 1 and -2 (PLC γ 1, PLC γ 2) and intracellular Ca²⁺ mobilization (Azzoni et al. 1992; Billadeau et al. 2003; Liao et al. 1993; McVicar et al. 1998; Ting et al. 1992). Analysis of PLC γ 2^{-/-} mice has demonstrated that PLC γ 2 is essential for all activating NK cell receptors to trigger granule exocytosis (Wang et al. 2000; Tassi et al. 2005). Studies with pharmacological inhibitors have strongly implicated the MAP kinase (MAPK) MAPK ERK1/2 and p38 in granule exocytosis (Chini et al. 2000; Trotta et al. 2000, 1998). Although multiple signaling pathways that lead to MAPK activation have been identified (Perussia 2000), how ERK1/2 and p38 elicit release of lytic granules remains to be determined. PI3K activates the NK cytolytic machinery by inducing sequential activation of the GTP-binding protein Rac1, followed by the kinases Pak1, MEK, and ERK1/2 (Jiang et al. 2000). In addition, PI3K generates phosphatidylinositol 3,4,5-trisphosphate (PIP₃), which mediates recruitment of PLC γ 1 and PLC γ 2 to the cell membrane and their activation (Deane and Fruman 2004; Koyasu 2003;

Okkenhaug and Vanhaesebroeck 2003). Surprisingly, mice lacking the p85 α regulatory subunit of PI3K do not exhibit major NK cell cytolytic defects. As PI3K includes multiple regulatory and catalytic subunits, redundancy may be in place. Like T and B lymphocytes, NK cells express intracellular adaptors such as the linker for activation of T cells (LAT), SLP-76, Gads, Grb2-associated binder 2 (Gab2), and 3BP2, which bridge together various components of signal transduction pathways. Although several signaling pathways have been shown to involve these adapters (Billadeau et al. 2003; Bottino et al. 2000; Chuang et al. 2001; Jevremovic et al. 1999, 2001; Klem et al. 2002; Zompi et al. 2004), it is not yet known whether any of them is required for NK cell activation.

6

Complexities in NK Cell Activation

6.1

Consequences of ITIM Phosphorylation

ITIM-bearing inhibitory NK receptors have been shown to recruit the protein tyrosine phosphatases SHP-1 and SHP-2 after ITIM phosphorylation (Colucci et al. 2002; Lanier 2003; McVicar and Burshtyn 2001; Vivier et al. 2004). In general, SHP-1 and SHP-2 dephosphorylate and deactivate multiple substrates that mediate NK cell activation, such as ITAM adaptors, protein tyrosine kinases, and Vav. However, SHP-1 and SHP-2 have different preferences for both phosphorylated ITIMs and substrates. Furthermore, each of them can either negatively or positively regulate signaling pathways depending on the experimental system studied. It is therefore not always trivial to ascribe a downregulating function to an ITIM-bearing receptor (see chapter by MacFarlane and Campbell, this volume).

6.2

2B4 Inhibition and Activation

In addition to ITIM-bearing inhibitory receptors, NK cells also express an unusual receptor, called 2B4, which binds CD48 on other cells. 2B4-mediated recognition of CD48 on target cells inhibits NK cell cytotoxicity (Lee et al. 2004), but 2B4-CD48 interaction between T cells and T cells or NK cells and NK cells enhances their activation (Lee et al. 2003). This intriguing dichotomy may be explained by the complexity of 2B4 signaling. 2B4 is a member of the CD2-like family of receptors, which also includes CD2, CD150, CD58, CD48, CD84, CD229, NTB-A, and CRACC (Engel et al. 2003; Nichols et al. 2005).

Some of these receptors, including 2B4, contain cytoplasmic immunoreceptor tyrosine-based switch motifs (ITSMs), which are distinct from ITAMs and ITIMs. Through ITSMs, CD2-like receptors bind a SH2 domain-containing cytoplasmic protein called SH2D1A (or SAP). SH2D1A recruits the Src kinase FynT, which phosphorylates the cytoplasmic tyrosines of the receptors. After tyrosine phosphorylation, CD2-like receptors sequentially recruit the SH2 domain-containing inositol-5 phosphatase-1 (SHIP-1), Shc, Dok1/2, and Ras-GAP, ultimately modulating MAPK activation (Veillette and Latour 2003).

Recently, it has been shown that cytoplasmic ITSMs, including those of 2B4, can also recruit a homolog of SH2D1A, called EWS-activated transcript 2 (EAT-2) (Morra et al. 2001b; Veillette and Latour 2003). EAT-2 may mediate a signaling cascade similar to that of SH2D1A. Alternatively, it could compete with SH2D1A for binding ITSM, thereby blocking the signaling cascade mediated by SH2D1A. Moreover, EAT-2 may block binding of other SH2-containing proteins, such as SHP-2, to ITSMs (Morra et al. 2001b). In this way, one current hypothesis is that the balance of SH2D1A and EAT-2 associated with the cytoplasmic domains of 2B4 and other ITSM-containing NK cell receptors determines the inhibitory or activating outcome of signaling. Importantly, mutations in SH2D1A cause X-linked lymphoproliferative disorder (XLP), a progressive combined variable immunodeficiency in which symptoms appear on Epstein-Barr virus (EBV) infection (Morra et al. 2001a; Nichols et al. 2005). Therefore, altered signaling by 2B4 and other ITSM-containing proteins expressed on NK cells may contribute to the pathogenesis of XLP. Understanding the function of SH2D1A and EAT-2 in NK cell inhibition and activation represents an important goal for NK cell research.

6.3

Roles of Adhesion

Early on in NK cell research, adhesion molecules were recognized as central players in NK cell-target cell and NK cell-matrix interactions as well as in NK cell effector functions (Helander and Timonen 1998). Mature NK cells constitutively express both β_1 and β_2 integrins. Their ligation results in rapid phosphorylation and activation of proline-rich tyrosine kinase 2 (Pyk2) (Gismondi et al. 2000), which regulates rearrangement of actin cytoskeleton through its constitutive association with paxillin. Pyk2 also contributes to NK cell activation by promoting ERK1/2 phosphorylation. Moreover, integrins activate a signaling cascade involving Vav1, Rac1, Pak1, and MKK3, ultimately leading to the activation of the MAPK p38 (Mainiero et al. 2000).

Recent evidence indicates that NK cells express a novel family of receptors, including DNAM-1, Tactile, and CRTAM, which bind a group of adhesion molecules called nectins and nectin-like molecules (Necls) (Boles et al. 2005; Bottino et al. 2003; Fuchs et al. 2004). Nectins and Necls are expressed on epithelial cells and mediate cell-cell adhesion (Sakisaka and Takai 2004). In addition, they are expressed on antigen-presenting cells (Boles et al. 2005). DNAM-1 binds Necl-5 and Nectin-2, Tactile binds Necl-5, and CRTAM binds Necl-2 (Boles et al. 2005; Bottino et al. 2003; Fuchs et al. 2004). The function of these adhesion interactions is under investigation. DNAM-1 triggers NK cell-mediated cytotoxicity (Shibuya et al. 1996) and has been reported to be physically and functionally associated with the β_2 integrin LFA-1, which induces DNAM-1 phosphorylation through the Src kinase Fyn-T (Shibuya et al. 1999). Thus DNAM-1-Necl-5/Nectin-2 interactions may be crucial in regulating NK cell adhesion to and lysis of target cells (Bottino et al. 2003). Tactile and CRTAM mediate strong adhesion but stimulate cytotoxicity weakly (Boles et al. 2005; Fuchs et al. 2004). Therefore, they may be preferentially implicated in NK cell migration into lymph nodes and peripheral tissues and/or NK cell proliferation and differentiation. Interestingly, Necl-2 is poorly expressed in epithelial tumors and overexpression of Necl-2 suppresses tumorigenesis of human tumor cell lines injected into nude mice (Kuramochi et al. 2001). This suggests that Necl-2 could be a major determinant of tumor immunogenicity, promoting anti-tumor NK cell responses through CRTAM (Boles et al. 2005).

6.4

Cytotoxicity and Cytokine Responses

Many NK cell ITAM-based receptors are capable of inducing full NK cell activation with intracellular Ca^{2+} mobilization, cytotoxic responses, and cytokine production (Moretta et al. 2001). However, signaling through certain NK cell receptors leads to more restricted effector function (see chapter by MacFarlane and Campbell, this volume). For example, stimulation of DAP10-linked NKG2D on NK cells leads to cytotoxicity but not IFN- γ secretion (Billadeau et al. 2003; Zompi et al. 2003). Alternatively, triggering of the unusual KIR family member KIR2DL4 has been found to result in IFN- γ secretion but not cytotoxicity (Rajagopalan et al. 2001). These findings illustrate how effector functions can be differentially triggered depending on the signaling pathway used and fit with their diverse roles in regulating and conducting immune responses.

7

A Challenge for the Future: Understanding the Integration of Signals by NK Cells

Although the reductionist analysis of NK cell signaling is rapidly leading to a detailed knowledge of individual pathways, it is essential to reconstruct the complexity of NK cell signaling and establish how these different components are integrated. In one experimental system, NK cell cytotoxicity has been tested against individual ligands expressed on a *Drosophila* insect cell line or directly coupled to beads (Barber et al. 2004). Remarkably, this approach has shown that expression of ICAM-1 on insect cells is sufficient not only to induce adhesion of NK cells to *Drosophila* cells through the β_2 integrin LFA-1 but also to induce activation signals that trigger lysis by NK cells. Coexpression of multiple activating and inhibitory ligands on *Drosophila* cells or beads will allow analysis of the relative contribution of the many different activating and inhibitory NK cell receptors (Barber and Long 2003).

Another powerful approach to reconstruct the complexity of NK cell signaling is three-dimensional immunofluorescence imaging (Davis 2002; Vyas et al. 2002). Interaction of NK cells with target cells leads to formation of an immunological synapse (IS) at the contact site. Molecules accumulating at the IS segregate in distinct domains. Segregated molecules are driven into specific arrangements depending on differences in cumulative activating and inhibitory signals. When inhibitory signals prevail over activating signals, SHP-1 clusters in the center of the cytolytic synapse, the Src kinase Lck has a multifocal distribution, and LFA-1 and LFA-1-associated talin form a ring that encloses SHP-1 and Lck. This inhibitory synapse is short-lived, the contact surface area shrinks rapidly, and deconjugation occurs within 2 or 3 min. In contrast, when activating signals overcome inhibitory signals, many activating molecules, such as protein kinase C- θ , WASP, Nck, SLP76, and LAT, as well as lytic granules, are recruited to the center of the IS. LFA-1 and talin form a peripheral ring, Lck maintains a multifocal distribution, and SHP-1 clusters in the periphery of the IS. This cytolytic IS is sustained and can last more than 15 min.

The complexity of NK cell signaling may go well beyond the integration of activating and inhibitory signals. Recent studies demonstrate that NK cells express multiple chemokine receptors, which trigger $G\alpha_i$ protein-mediated signals when engaged by constitutive or inflammatory chemokines (Maghazachi 2003). Moreover, NK cells express Toll-like receptors, which can sense pathogen components and trigger cytokine secretion (Chalifour et al. 2004; Hornung et al. 2002; Sivori et al. 2004). Finally, there is abundant evidence that IL-2, which is commonly used to culture NK cells, has profound effects

on NK cell signaling, potentiating alternative cytotoxicity pathways that may not operate in freshly isolated NK cells. For example, WASP deficiency affects cytotoxicity of fresh but not IL-2-cultured NK cells (Gismondi et al. 2004). Additional cytokines, such as IFN- α , IL-12, IL-23, IL-15, and IL-21, influence NK cell maturation and/or acquisition of effector functions (Bonnema et al. 1994; Nguyen et al. 2002; Nutt et al. 2004; Vosshenrich et al. 2005). Understanding how these signaling pathways are integrated in vivo during NK cell-mediated immunosurveillance against viruses and tumors represents a challenging goal for NK cell research in the near future.

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