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# Strategies for target cell recognition by natural killer cells

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**Summary:** Stimulation of natural killer (NK) cells is regulated by a complex balance of inhibitory and stimulatory receptors expressed by NK cells. However, the interaction of stimulatory receptors and their ligands is poorly understood. One stimulatory receptor, NKG2D, is expressed by all NK cells, stimulated $CD8^+$ T cells, γδ T cells and macrophages. Recently, progress has been made in defining cellular ligands for NKG2D. Four different families of ligands have been identified in mice and humans, all of which are distantly related to MHC class I molecules. Some of the ligands are upregulated in transformed and infected cells, provoking an attack by the innate and adaptive immune systems. It appears that these "induced-self" ligands recognized by the NKG2D receptor may be a precedent for a new strategy of target cell recognition by the immune system.

## Introduction

Natural killer (NK) cells are bone marrow-derived, large granular lymphocytes that recognize and lyse a variety of transformed and infected cells (1–5). In addition to their cytotoxic effector functions, NK cells are also very potent producers of cytokines (including interferon (IFN)-γ, tumor necrosis factor (TNF)-α and granulocyte–macrophage colony-stimulating factor (GM-CSF)) and chemokines (such as macrophage inflammatory protein (MIP)-1 family members and RANTES) (for an overview see (6)). NK-cell responses occur rapidly (within hours), providing potent effector activities before the adaptive immune response is sufficiently developed to control the infection or tumor.

### Inhibitory target cell recognition

Unlike B and T cells, NK cells do not require gene rearrangement machinery to assemble their receptor genes. However, NK cells do clearly discriminate among potentially harmful cells (i.e. tumor cells, infected cells and stressed cells). Fifteen years ago a model was first introduced proposing that NK cells discriminate target cells based on the levels of target cell self-MHC class I expression (7). Low or absent self-MHC class

I molecules would activate the NK cell, whereas normal levels of self MHC class I would inhibit them. This model was supported by studies with genetically engineered mice, which showed that NK cells attack otherwise normal cells that lack some or all self-MHC class I molecules (8, 9). In further support of the model, three families of inhibitory, MHC class I-recognizing receptors expressed by NK cells were subsequently discovered. The first to be discovered were the Ly49 receptors in rodents, which are related in structure to C-type lectins and which bind directly to MHC class Ia molecules (10–13). In humans only a single apparently non-functional *Ly49* gene has been identified (14, 15). Human NK cells express an alternative set of structurally different receptors, the killer cell immunoglobulin-like receptors (KIR), which also bind directly to MHC class Ia molecules (16–19). So far no KIR receptors have been identified in rodents. More recently another lectin-like family of receptors has been characterized, which is conserved in primates and rodents and consists of the CD94/NKG2 heterodimers (20–22). One of several NKG2 isoforms (NKG2A, B, C, E; NKG2A and B represent alternative splice variants encoded by the same gene) can associate with the unique CD94 chain (21, 22). Of these isoforms, only CD94/NKG2A and B are known to be inhibitory receptors. Like the other inhibitory receptors, the CD94/NKG2A receptor also detects MHC class Ia expression by target cells, but in this case the mode of MHC class Ia detection is indirect: CD94/NKG2A identifies class Ia-positive cells by specifically recognizing a cleaved peptide from the leader sequences of class Ia molecules, bound into the groove of a class Ib molecule. The class Ib molecule is Qa-1 in mice and HLA-E in humans (23–25).

The cytoplasmic domains of all inhibitory NK-cell receptors contain an immunoreceptor tyrosine-based inhibitory motif (ITIM) with the consensus sequence (I/VxYxxL/V). Upon tyrosine phosphorylation (possibly by src-family kinases), the src homology 2-containing tyrosine phosphatases (SHP)-1 and SHP-2 are recruited to these receptors (26, 27). The precise sequence of biochemical events that subsequently results in the inhibition of NK cell function remains to be defined.

The various MHC-specific inhibitory receptor genes are expressed by overlapping subsets of NK cells such that an individual cell expresses a few inhibitory receptors. This leads to a complex combinatorial repertoire of NK specificities for MHC class I molecules. Evidence suggests that the repertoire is further shaped by education mechanisms that create a useful and self-tolerant repertoire. The formation of the NK-cell repertoire is the subject of a recent review from our group (28), and will not be addressed in detail here. In brief, it appears that the inhibitory receptor repertoire is structured to prevent NK cells from lysing self cells that express normal levels of each self class Ia molecule, but to unleash NK cells in response to target cells that downregulate some or all class Ia molecules. This may well be of great biological significance, as MHC downregulation often occurs in transformed and infected cells.

While inhibitory recognition of class I MHC molecules accounts in part for the target cell specificity of NK cells, it has been recognized that some sensitive target cells express normal levels of MHC class I molecules, while some other cells are not sensitive to NK-cell lysis despite low or absent MHC class I expression. Thus, it is clear that NK cells employ additional mechanisms to discriminate target cells. It has long been proposed that NK cells also make use of stimulatory receptors, specific for unknown ligands, that may be differentially expressed by target cells. While candidate stimulatory receptors have been identified over the years, the ligands for most of these receptors have not been identified. Consequently, an understanding of the principles governing stimulatory recognition by NK cells has lagged behind our understanding of inhibitory recognition. Recently however, significant progress has been made in elucidating the biological function of some of these receptors. In the following sections we will summarize some general aspects of stimulatory NK-cell receptors and then review in some detail the best characterized stimulatory receptor–ligand system to date, consisting of the NKG2D receptor and its ligands.

## Positive target cell recognition: stimulatory receptors and their ligands

Stimulatory NK-cell receptors can be roughly divided into one group of receptors that recognizes MHC class I molecules and another group that does not (*Table 1*). The MHC class I-specific stimulatory receptors include members of each family that also encodes inhibitory receptors (Ly49, KIR or CD94/NKG2). The presumptive non-MHC-specific stimulatory receptors include the lectin-like NKG2D (though one of its ligands is arguably a non-classical class I protein, see below), NKR-P1A and NKR-P1C receptors, and the Ig-like NKp46, NKp44 and NKp30 receptors (for a more complete list see *Table 1*). A third group of receptors mentioned in *Table 1*, CD2, CD16, CD28, CD40L, 2B4, DNAX accessory molecule (DNAM)-1/leukocyte function-associated antigen (LFA)-1 and Lag-3, are most likely to co-activate rather than directly stimulate NK cells. Below we will focus on the first two groups of receptors.

***Table 1*. Stimulatory receptors on NK cells**

| | Ligand(s) | Adapter | Species | Expression pattern | References |
|---|---|---|---|---|---|
| Stimulatory receptors recognizing MHC class I molecules | | | | | |
| Ly49D | H2-D$^d$ | KARAP/DAP12 | m | Variegated | 39, 41, 61 |
| Ly49H | Unknown | KARAP/DAP12 | m | Variegated | 40, 48 |
| NKG2C, NKG2E | Qa-1$^b$ – HLA-E | KARAP/DAP12 | m, r, h | Variegated | 20, 23, 25, 46, 119 |
| KIR2DS | HLA-C | KARAP/DAP12 | h | Variegated | 43–45 |
| KIR3DS | ? – HLA molecule | KARAP/DAP12 | h | Variegated | 43–45 |
| Stimulatory receptors recognizing non-MHC class I molecules | | | | | |
| NKG2D | Rae1α, β, γ, (δ)<br>H60<br>MICA, MICB<br>ULBP1, 2, 3 | KAP/DAP10 | m, r, h | All NK cells,<br>all CD8$^+$ T cells (after T-cell receptor (TCR) stimulation in mice),<br>all macrophages (after stimulation),<br>ca. 50% of NKT cells, γδ T-cell subset | 20, 31, 33, 78–82, 92 |
| NKp46 | ? Viral hemagglutinin | CD3ζ, FcεRγI | m, h | All NK cells | 49, 50, 56, 57, 121 |
| NKp44 | Unknown | KARAP/DAP12 | h | All NK cells (after IL-2 stimulation) | 51, 52 |
| NKp30 | Unknown | CD3ζ, FcεRγI | h | All NK cells | 53 |
| NKR-P1A | Unknown | Unknown | m, r, h | Subset | 65, 77 |
| NKR-P1C | Unknown | FcεRγI | m, r | All (or most) NK cells | 62–68, 74–76 |
| CD16 | IgGs | CD3ζ, FcεRγI | m, r, h | Most NK cells | 122–126 |
| CD2 | CD58 (LFA-3),<br>? CD48 ? | CD3ζ<br>(no direct association) | m, r, h | Most NK cells | 127–129 |
| CD244 (2B4) | CD48 | SAP (SH2D1A) | m, h | Most or all NK cells (also CD8$^+$ αβ T cells and γδ T cells and myeloid cells) | 130–136 |
| CD28 | B7.1/B7.2 | YxxM in cytoplasmic domain | m, h | Mice: splenic NK cells<br>Humans: only on fetal NK cells | 137–142 |
| CD40L | CD40 | | m, h | Humans: induced on all NK cells<br>Mice: ? | 143, 144 |
| LFA-1/DNAM-1 | Ligands for LFA-1 (ICAM-1, 2, 3) | DNAM-1 recruits the protein tyrosine kinase *fyn* | h | Human: most NK cells, αβ T cells , γδ T cells, monocytes and some B cells<br>Mice: ? | 145–147 |
| Lag-3 | MHC class II | associates with the CD3–TCR complex | m, h | NK cells, activated CD4$^+$ and CD8$^+$ T cells | 148–150 |

A common feature of all stimulatory receptors is that they lack the ITIM motif in their cytoplasmic domains. Instead, all known stimulatory receptors have charged residues in their transmembrane domains that are necessary for association with adapter signaling proteins, which have very short extracellular domains and are not believed to participate in ligand binding. Instead, the intracellular domains of the adapter proteins have docking sites for downstream stimulatory signaling molecules. Most adapters (FcεRγI, CD3ζ and the killer cell-activating receptor-activating protein (KARAP)/DAP12 molecule) contain immunoreceptor tyrosine-based activation motifs (ITAM) in their cytoplasmic domains, which allows them to associate with ZAP70 and/or syk-family kinases (29, 30). A recently defined adapter protein, designated KAP10 or DAP10, has a YxxM motif in its transmembrane domain, which allows recruitment of phosphatidylinositol (PI) 3-kinase (31, 32). So far, the only known receptor that associates with KAP/DAP10 is NKG2D (31, 33). The NKR-P1 proteins associate with FcεRγI (34), but the rodent NKR-P1s also have a CxCP motif in their cytoplasmic domain, which has also been found in the cytoplasmic domains of CD4 and CD8 and is thought to interact with p56$^{lck}$ (35). One report demonstrated a direct interaction of rat NKR-P1 and p56$^{lck}$ (36). The human NKR-P1A lacks the CxCP motif (37, 38).

*Stimulatory receptors recognizing MHC class I molecules*

Each family of inhibitory receptors contains MHC class I-specific stimulatory receptors. In the Ly49 family these are the Ly49D and Ly49H receptors (*Table 1*) (39, 40). The ligand for Ly49D is the H2-D$^d$ molecule (41). A class I specificity for Ly49H has not yet been assigned. NKG2C and NKG2E are stimulatory members of the CD94/NKG2 family (*Table 1*) (20, 25, 42). They recognize (as do their inhibitory counterparts) the Qa-1 molecule in mice (25) and the HLA-E molecule in humans (23). The "short" KIR (KIR2DS and KIR3DS) are stimulatory members of the KIR family (43–45). KIR2DS rec-

ognizes HLA-C, while the specificity of KIR3DS is currently unclear. Interestingly, all these stimulatory counterparts of the inhibitory NK-cell receptors use a single adapter protein, KARAP/DAP12 (25, 30, 40, 46), allowing interactions with ZAP70 and syk-family kinases.

In contrast to the non-MHC class I-specific stimulatory receptors but very similar to the inhibitory receptors, the MHC class I-specific stimulatory receptors are expressed in a variegated and predominantly stochastic fashion by subsets of NK cells ((39, 47, 48), reviewed in (28)). To date, an understanding of the adaptive value of NK-cell stimulatory receptors specific for MHC class Ia ligands remains elusive.

*Stimulatory receptors recognizing non-MHC class I molecules*

Several stimulatory NK-cell receptors with no apparent specificity for MHC class I molecules have been reported (*Table 1*). However, in most cases the cellular ligands for these receptors have yet to be identified and the biological function of these receptors remains unclear. In contrast to the MHC class I-recognizing stimulatory receptors, these stimulatory receptors are expressed by most or all NK cells.

Recently, Moretta and colleagues identified three Ig-like stimulatory NK-cell receptors expressed by human NK cells, NKp46 (49, 50), NKp44 (51, 52) and NKp30 (53), that are involved in the recognition of various tumor cell lines. The NKp30 cDNA had been described earlier as a protein of unknown function encoded in the TNF cluster of the human MHC complex and had been assigned the name 1C7 (54, 55). NKp46 and NKp30 are constitutively expressed by all human peripheral blood NK cells, and their expression seems to be confined to the NK-cell compartment (49, 53). NKp44 is not expressed by resting human NK cells but is upregulated by all NK cells after stimulation with interleukin (IL)-2 (51). NKp44 was also detected on some human $\gamma\delta$ T-cell lines (51). In the case of NKp46, a mouse homolog has been identified and has been named mouse activating receptor-1 (MAR-1) (56). For signaling, NKp46 and NKp30 associate with $Fc\varepsilon R\gamma I$ and $CD3\zeta$, whereas NKp44 associates with KARAP/DAP12 (52, 53). Triggering of any of these receptors resulted in the induction of cytotoxic activity and intracellular $Ca^{2+}$ mobilization (49, 53). Stimulation of IFN-$\gamma$ and TNF-$\alpha$ production after receptor cross-linking could only be demonstrated for NKp46 (49). Cytotoxicity against various tumor cell lines was significantly blocked by including monoclonal antibodies specific for one or a combination of these receptors in the assay, suggesting that these tumor cells express ligands for NKp46, NKp30 or NKp44 (50). Taken together, the available data suggest an important role for these receptors in recognizing tumor cells. Very recently, a provocative report by Mandelboim et al. reported that human NKp46 directly recognizes viral hemagglutinins, including the influenza virus hemagglutinin and the Sendai virus hemagglutinin neuraminidase (57). Furthermore, expression of viral hemagglutinins on target cells induced target cell lysis by human peripheral blood NK cells and NK cell clones. Mandelboim et al. could not detect binding of soluble NKp46 to the Epstein–Barr virus-transformed B-cell line LCL721.221, though lysis of 721.221 cells was reportedly inhibited by a monoclonal antibody to NKp46 (50, 51). It remains to be directly determined whether tumor cells express ligands for NKp46.

All of the MHC class I-specific stimulatory receptors, as well as the NKp44 receptor, use the KARAP/DAP12 molecules for signaling (*Table 1*). Recently, two groups reported loss of function mutants of KARAP/DAP12 (58, 59). The development of NK cells is unaltered in these mice. Tomasello et al. used a "knock-in" strategy to functionally inactivate the KARAP/DAP12 signaling pathway. This strategy preserved the expression of KARAP/DAP12 as well as the interaction with and expression of associated receptors. Using this approach, the effects of abolishing KARAP/DAP12 signaling on the formation of the inhibitory receptor repertoire in NK cells could be examined without abolishing cell surface expression of the relevant stimulatory receptors. The mutation did not alter the number of cells expressing all tested Ly49 and NKG2/CD94 receptors, nor the cell surface levels of these receptors (58). However, redirected lysis of $FcR\gamma^{+}$ target cells mediated by a monoclonal antibody specific for Ly49D was totally abrogated in NK cells from the mutant mice. Furthermore, NK-cell lysis of Chinese hamster ovary tumor cells, which is reported to be partially dependent on Ly49D (60, 61), was greatly reduced in KARAP/DAP12-deficient mice (58, 59). Interestingly, NK-cell lysis of other tumor targets (YAC-1, Bw15.02, RMA, RMA/S, J774) was completely unaffected by the KARAP/DAP12 mutations. Surprisingly, inactivation of KARAP/DAP12 led to a dramatic increase of dendritic cells in mucosa and skin (58) and a striking impairment in the priming of $CD4^{+}$ T cells (59) and $CD8^{+}$ T cells (58). The latter results suggest that KARAP/DAP12 has non-redundant functions in a non-NK-cell compartment.

The prototype mouse NK-cell antigen NK1.1 is encoded by the *Nkr-p1c* gene (62, 63). Three highly related genes, *Nkr-p1a*, *Nkr-p1b* and *Nkr-p1c*, have been identified in mice and rats (64–69). NKR-P1A and NKR-P1C are stimulatory isoforms, whereas NKR-P1B is an inhibitory isoform (69). Only a

single human NKR-P1 homolog has been identified to date (37). The *Nkr-p1* genes map to mouse chromosome 6 (70), human chromosome 12p12-p13 (37, 71) and rat chromosome 4 (72) in a region that has been designated the "NK gene complex" (73). Mouse NKR-P1C associates with FcεRγI for downstream signaling (34). Additionally, rodent NKR-P1 proteins have a CxCP motif in their cytoplasmic domain providing a docking site for $p56^{lck}$ (35, 36). Cross-linking of NKR-P1C activates NK cells (74–76). Analysis of a mutant NK-cell line suggested that NKR-P1 receptors participate in recognition of certain tumor cell lines (77). The physiologically relevant ligands for NKR-P1 receptors remain unknown, however.

### The NKG2D receptor

The best characterized stimulatory NK receptor system in mouse and human is the NKG2D receptor. Recent progress in understanding this receptor system has come from the identification of cellular ligands by our group and others (33, 78–80). The lectin-like type II transmembrane receptor NKG2D was first identified 10 years ago as a human cDNA clone in the same study that yielded the first NKG2A, B and C cDNAs (20). Subsequently, we and others identified the murine NKG2D homolog (81–83). The *Nkg2d* gene is localized in the mouse NK gene complex (chromosome 6) between the *Cd94* and *Nkg2e* genes (82). Despite its name and its genomic location in a cluster of *Nkg2* genes, NKG2D differs dramatically in sequence from the other NKG2 proteins (only 24.5% identity in amino acid sequence), does not associate with CD94 (31) (Diefenbach & Raulet, unpublished data), and does not recognize Qa-1/HLA-E. It therefore appears that the common designation of NKG2 for all these proteins is misleading.

Very recently an unligated X-ray crystal structure of mouse NKG2D was reported (84). NKG2D retains an overall structure that is surprisingly homologous to other members of the C-type lectin family, including CD94, Ly49A, rat MBP-A and CD69, despite relatively low sequence identity. The NKG2D monomer contains two β-sheets, two α-helices and four disulfide bonds. The most obvious difference between NKG2D and the other C-type lectins is a newly identified β-strand (named 5′), which is part of loop 5. Compared to the other C-type lectins, the loops 5′ and 5 in NKG2D are conformationally restricted due to the intervening 5′ β-strand. This could have consequences in ligand binding (84). Interestingly, an analysis of the surface electrostatic potentials revealed a very extensive central electropositive surface stripe that spans the dimer interface as well as flanking electropositive patches. Intriguingly, the top surfaces (relative to membrane location) of the human MHC class I chain-related (MIC) A structure (85) as well as the predicted MICB structure (84) revealed a complementary electrostatic profile that may serve as the interacting surface with NKG2D. MICA and MICB are ligands for NKG2D (see below).

### The KAP/DAP10 adapter

NKG2D has two charged residues in the deduced transmembrane domain leading to the prediction that NKG2D is a stimulatory receptor that uses these residues to associate with an adapter molecule (81, 82). However, none of the known adapter molecules involved in NK-cell signaling (i.e. FcεRγI, CD3ζ and KARAP/DAP12) associate with NKG2D (31). Two recent reports described a new adapter molecule designated DAP10 (31) or KAP10 (32). Interestingly, the *Kap/Dap10* gene and the *Dap12* gene are localized less than 150 bp apart in opposite transcriptional orientations on human chromosome 19q13.1 and mouse chromosome 7 (31, 59). KAP/DAP10 is expressed in several human NK-cell lines, a myeloid cell line (U937) and in $CD8^{+}$ T cells (32). Wu et al. demonstrated that KAP/DAP10 associates with NKG2D on the surface of human NK-cell lines (31, 86). In contrast to the other known adapters for NK-cell signaling, KAP/DAP10 does not have a consensus ITAM motif in its cytoplasmic domain. Instead, the KAP/DAP10 cytoplasmic domain has a YxxM motif, which has been shown to recruit the p85 subunit of PI 3-kinase (31, 32, 87). After NKG2D cross-linking, KAP/DAP10 is phosphorylated. Phosphorylated KAP/DAP10 is able to recruit the p85 subunit of PI 3-kinase, resulting in the activation of Akt (32). Chang et al. showed that KAP/DAP10 also interacts with another adapter protein, Grb2 (32). Interestingly, the intracellular domain of CD28 also has a YxxM motif, suggesting that the NKG2D-KAP/DAP10 receptor complex has a co-stimulatory as opposed to a primary triggering function (86). According to a recent report, signaling via PI 3-kinase plays a pivotal role in the pathway that triggers cytotoxicity of NK cells (88). Pharmacological inhibition of PI 3-kinase blocked killing by preventing mobilization of perforin and granzyme B to the cell–cell interface between NK92 cells (an NK line) and Raji target cells. The authors implicated the Rho-family GTP-binding protein Rac1 and the p21-activated kinase 1 (PAK1) as downstream targets of PI 3-kinase with Rac1 being upstream of PAK1. Expression of a dominant negative Rac1 or a kinase-deficient PAK1 mimicked the effect of pharmaco-

logical inhibition of PI 3-kinase, whereas constitutively active Rac1 rescued killing mediated by cells treated with PI 3-kinase inhibitors (88). Downstream of PAK1 is the extracellular-signal-regulated kinase (ERK) mitogen-activated protein kinase (MAPK) (88, 89). Currently it is not clear whether this intracellular signaling pathway is a general one for triggering NK cytotoxicity or is specific to the undefined receptor involved in the NK92-Raji target cell interaction. Another report showed that the Rac1 pathway can be activated by Vav (90, 91). Activation of Vav is thought to be triggered by src- or syk-family protein tyrosine kinases, which are involved in signaling by Fc receptors and the B and T-cell antigen receptors. The identity of the upstream receptor systems that activate PI 3-kinase and Vav remain unclear. However, the role of PI 3-kinase in triggering cytotoxicity, in combination with the finding that the KAP/DAP10 receptor system recruits and activates PI 3-kinase, suggests that NKG2D may serve as a major triggering receptor for cytotoxicity.

### NKG2D receptor expression

The expression and regulation of NKG2D has not been fully investigated. However, it is clear from the available data that NKG2D is not restricted to NK cells but functions as well in other cell types. In addition, there seem to be significant differences in the expression pattern between mice and humans. In mice and humans, the NKG2D receptor is constitutively expressed by all NK cells (33, 78). It appears that expression of NKG2D by NK cells is not markedly regulated by stimulating cytokines such as IL-12, IFN-$\gamma$, IFN-$\alpha/\beta$ or IL-2 (33). Little has been reported concerning the regulation of NKG2D expression by human NK cells.

Importantly, certain T-cell populations also express NKG2D. In mice, resting conventional CD4 and CD8 T cells do not express significant levels of NKG2D, but the receptor is strongly upregulated by T-cell receptor cross-linking in all $CD8^+$ but not $CD4^+$ T cells (33). Co-stimulation with anti-CD28 did not further increase NKG2D expression by $CD8^+$ T cells. In line with these data, we observed upregulation of NKG2D expression by $CD8^+$ T cells responding *in vivo* at the peak of an infection with lymphocytic choriomeningitis virus (C. W. McMahon, A. Diefenbach, D. H. Raulet, unpublished observations). These findings indicate that NKG2D is induced in mouse $CD8^+$ T cells activated through the T-cell receptor. In contrast, it is reported that all human $CD8^+$ T cells express NKG2D constitutively, without activation (78, 92). As in mice, $CD4^+$ T cells do not express NKG2D (78).

In mice and humans, many $\gamma\delta$ T cells express NKG2D. In mice, NKG2D expression appears to be primarily restricted to the subset of $\gamma\delta$ T cells that expresses CD44 (A. M. Jamieson, A. Diefenbach, D. H. Raulet, unpublished data), whereas in humans, it is reported that all $\gamma\delta$ T cells express NKG2D (78). Many mouse CD1-restricted T cells also express NKG2D (A. M. Jamieson, A. Diefenbach, D. H. Raulet, unpublished data), but comparable analysis of human CD1-restricted T cells has not been reported.

Finally, we presented data showing that NKG2D is expressed by activated macrophages in mice (33). Whereas resting peritoneal macrophages did not express NKG2D, stimulation with lipopolysaccharide (LPS), IFN-$\gamma$ or IFN-$\alpha/\beta$ strongly upregulated NKG2D protein and mRNA in these cells (33) (Diefenbach & Raulet, unpublished data). To date, it is not clear whether human myeloid lineage cells also express NKG2D after appropriate stimulation.

### Ligands for NKG2D

Recently, several families of ligands have been described for NKG2D, all of which are distantly related to MHC class I molecules. The identification of these ligands has allowed a more detailed analysis of this receptor system than of any other stimulatory NK-cell receptor.

### Ligands for human NKG2D

#### The MIC family of proteins

Bauer et al. found that a soluble multimeric version of the MHC class I-like molecule MICA binds to virtually all NK cells. The structure recognized by MICA was identified as human NKG2D (78). The *MICA* and the closely related *MICB* genes are located near HLA-B in the human MHC (93). The MIC proteins contain three MHC-like domains, but in contrast to class Ia and most class Ib MHC molecules they do not associate with $\beta_2$-microglobulin and probably do not bind peptide (85). Under normal circumstances, the expression of MIC proteins is highly restricted to the human intestinal epithelium (gastric, small and large intestine). Additionally, in the subcapsular cortex of infant thymi a population of stellate MIC expressing epithelial cells could be identified (94). Interestingly, the 5′ flanking regions of the MIC genes reportedly contain heat shock promoter elements similar in some respects to those in the *Hsp70* promoter (94). Indeed, exposure of a MIC-expressing tumor cell line (HeLa) to heat shock led to a moderate increase of MIC mRNA (94). More significantly, high MIC expression could be detected on many

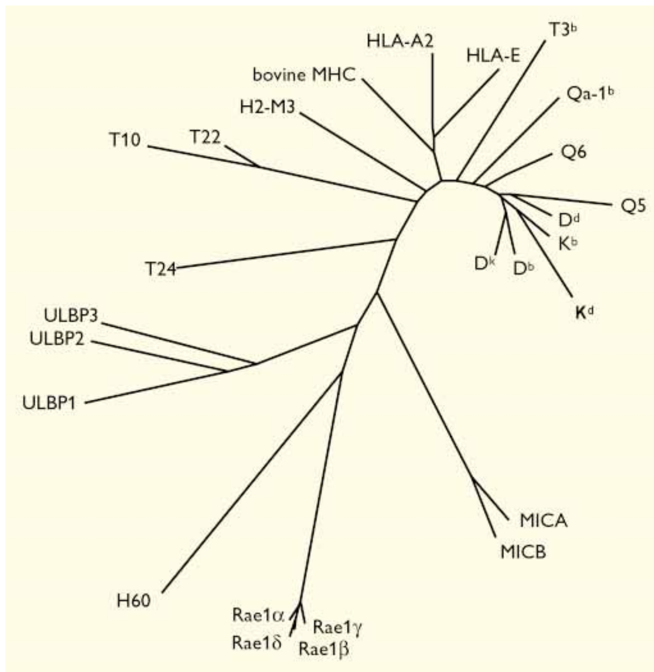


**Fig. 1. Relationship between NKG2D ligand families and various MHC class I molecules.** The tree dendrogram illustrates the relationship between the four families of mouse and human NKG2D ligands and classical and non-classical MHC class I molecules. The distance from each end-point of the tree to another end-point is a measure of the similiarity of the sequences of two compared proteins. Sequences from the (NCBI database) were aligned with Clustal W, and the dendrogram was generated with Phylip 3.572.

human epithelial tumors (95). Recently, Groh et al. demonstrated that cultured fibroblasts and endothelial cells infected with cytomegalovirus (CMV) strongly upregulated MIC expression (92). Increased MIC expression could also be detected in lung specimens with interstitial pneumonia caused by CMV (92). These data suggested that MIC proteins are upregulated in response to cellular stress, transformation and/or viral infection. MIC expression can thus be viewed as an identification system that cells use to provoke attack by the immune system.

### The family of UL16-binding proteins (ULBPs)

A recent report characterized a second family of ligands for human NKG2D (80). The authors identified cellular proteins that bind to the human CMV protein UL16, which is a type I transmembrane protein known to be expressed by CMV-infected cells. They expression-cloned a novel family of cDNAs that includes three members, designated ULBP1, 2 and 3. The deduced amino acid sequences of the three ULBPs are 55–60% identical (*Fig. 1*). The extracellular domain structure shows similarity to the $\alpha_1$ and $\alpha_2$ domains of MHC class I molecules and comprises a overall amino acid identity of ≈25% to human HLA molecules (*Fig. 1*). The ULBPs are glycosylphosphatidyl inositol (GPI)-anchored cell surface proteins. It is noteworthy that soluble UL16 binds to ULBP1, ULBP2 and MICB but not to ULBP3 and MICA (80). As assayed by RT-PCR, ULBP mRNA was detected in a wide range of tissues, including heart, brain, lung, liver, testis, lymph node, thymus, tonsil and bone marrow. ULBP transcripts were also abundant in fetal heart, brain, lung and liver. However, in some tissues/cells where high ULBP mRNA was detected, such as the Raji cell line, no cell surface ULBP expression was detected with a monoclonal antibody (80). While additional studies will be necessary to understand how ULBPs are regulated, the current data raise the possibility that ULBP cell surface expression is regulated in part at a post-transcriptional level. Employing soluble (s) ULBPs, Cosman et al. demonstrated that sULBPS bind to all human NK cells and to a human NK-cell line. Further analysis demonstrated that the soluble ligands bind to human NKG2D. In addition, exposure of NK cells either to sULBPs or to cells expressing ULBPs resulted in activation of the NK cells via engagement of NKG2D (80). An interesting hypothesis is that UL16 expressed by CMV-infected cells may protect the infected cells from NKG2D$^+$ effector cells by masking cellular ULBPs expressed by the same cell (80). UL16 may therefore provide CMV with a potent immune evasion strategy to prevent lysis of infected cells by NK and CD8$^+$ T cells.

## Ligands for mouse NKG2D

Interestingly, despite considerable efforts, no MIC homologs have been identified in mice. The syntenic region of the mouse MHC contains fewer genes, and it is possible that no functional orthologs of the MIC genes exist in this region (96). Alternatively, it remains possible that a murine functional ortholog exists, but exhibits little direct sequence identity with MIC; this situation was observed in the case of HLA-E versus Qa-1, which exhibit no more overall sequence similarity than any mouse–human MHC pair, but clearly carry out the same function (*Fig. 1*) (24).

Employing fluorescently labeled, soluble tetrameric NKG2D we (33) and others (79) recently expression cloned ligands for the mouse NKG2D receptor. Surprisingly, two related families of proteins were identified as ligands, each previously cloned but of unknown function: the retinoic acid early-1 (Rae1) proteins (97–99) and H60, a minor histocompatibility antigen (100).

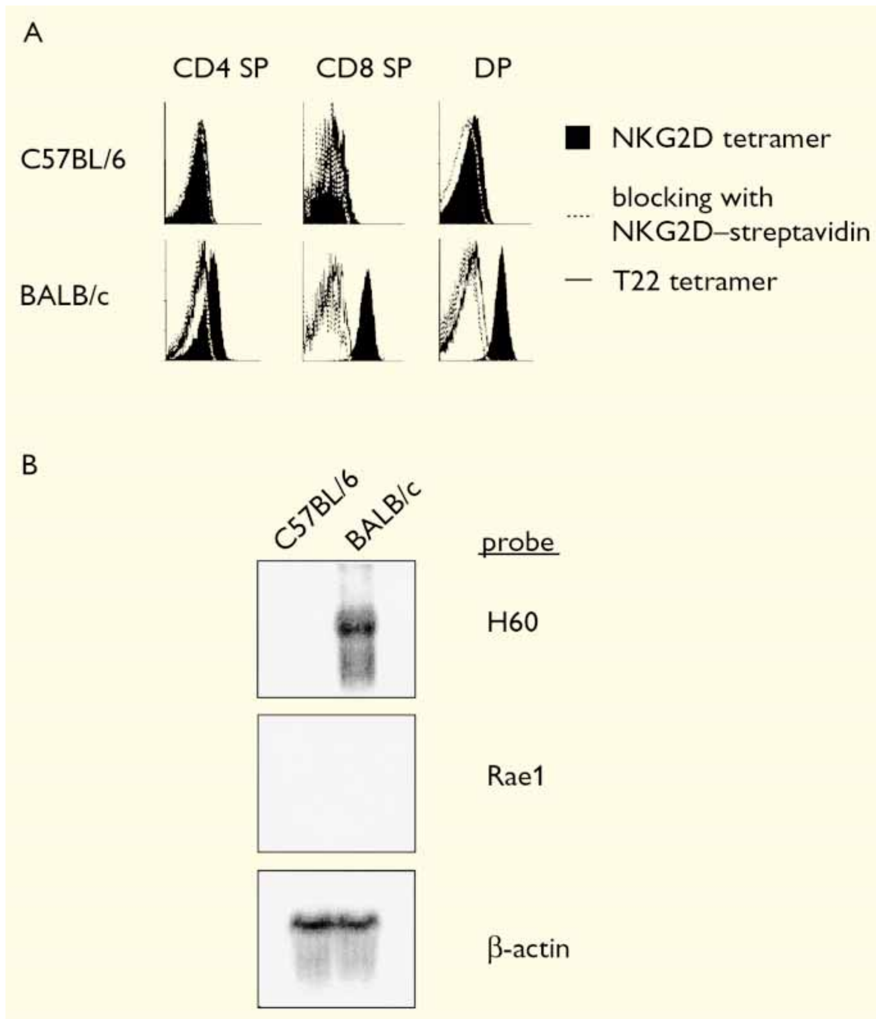


**Fig. 2. Expression of the H60 NKG2D-ligand by thymocytes from BALB/c mice.** **A.** Freshly isolated thymocytes from C57BL/6 and BALB/c mice were stained with monoclonal antibodies to CD4 and CD8 and with a streptavidin–phycoerythrin-complexed NKG2D tetramer (filled histograms). Staining with an irrelevant tetramer (solid line) and blocking of the NKG2D tetramer staining (dashed line) with an excess of unlabeled tetramer was performed to show the specificity of the staining. The histograms show electronic gating on the designated cell populations. **B.** Total thymocyte RNA (20 μg) from C57BL/6 and BALB/c mice was fractionated by electrophoresis in an 0.8% agarose gel and immobilized on a charged nylon membrane. The membrane was hybridized with a full length H60 cDNA probe (top panel), rehybridized (after stripping of the membrane) with a Rae1β probe (middle) and finally (after restripping of the membrane) hybridized with a β-actin probe as a loading control (bottom). [*Fig.* 2*A* reproduced from Diefenbach A, Jamieson AM, Liu SD, Shastri N, Raulet DH. Ligands for the murine NKG2D receptor: expression by tumor cells and activation of NK cells and macrophages. Nat Immunol 2000;**1**:119–126.]

### The Rae1 family of proteins

There are four highly related members of the Rae1 family (Rae1α–δ), each encoded by a separate gene (*Raet1a–d*) (79, 97, 98). The Raet1 cDNAs were originally cloned based on the inducibility of one member in F9 embryocarcinoma cells by retinoic acid (97, 98). Retinoic acid failed, however, to induce Rae1 expression in selected Rae1-negative tumor cell lines that we tested (Diefenbach & Raulet, unpublished data). Interestingly, Rae1 mRNAs were not detected in various normal adult tissues (98). Nevertheless, Rae1 mRNA is frequently upregulated in mouse tumor cell lines (33) (Diefenbach & Raulet, unpublished data). Rae1 mRNA is also expressed at low levels throughout embryos at day 9 of gestation and in the brain/head region of day 10–14 embryos, raising the possibility that Rae1 plays a role in embryonic development (98, 99). The available data suggest that Rae1 expression is extinguished after the embryonic stages, only to be re-expressed during the process of malignant transformation to tag these cells for attack by the immune system.

### The H60 minor histocompatibility antigen

H60 is encoded by a unique gene, which encodes a dominant minor histocompatibility antigen in the response of C57BL/6 T cells to BALB.B cells (100). The response to H60 is a typical MHC-restricted response in which the LTFNYRNL peptide, which is located near the N-terminus of H60, is presented by the $K^b$ MHC class I molecule (100). H60 expression was not detected in any cells tested from adult C57BL/6 mice, although an *H60* gene can be detected in these mice by genomic Southern blotting (N. Shastri, unpublished data). H60 expression was detected in Con A or LPS lymphoblasts (mainly by $CD4^+$ T cells and $CD19^+$ B cells) (33, 100) and at high levels on most BALB/c thymocyte subsets, especially CD8 single positive and double positive cells (*Fig.* 2) (33).

Previous studies mapped both *H60* and *Raet1* genes to murine chromosome 10, raising the possibility that these genes comprise a related gene family (97, 100). Although H60 and Rae1 proteins exhibit a similar domain structure, the amino acid sequences are only distantly related (*Fig. 1*), exhibiting an overall amino acid identity of 25.2% and a similarity of 39.1% (33). The extracellular segment of both proteins shares similarity with the $\alpha_1$ and $\alpha_2$ domains of MHC class I molecules (33, 99). Most Rae1 is GPI anchored to the membrane (33, 79, 98) whereas H60 shows no evidence of GPI linkage and may be a typical transmembrane protein (33).

These novel ligands can not be considered homologs of MIC, because their chromosomal location is non-syntenic, they exhibit a different domain structure, and are considerably divergent in sequence from MIC proteins (*Fig. 1*). However, the human *ULBP* genes exhibit a similar domain structure, and are located on human chromosome 6 in a region that is syntenic to the region of mouse chromosome 10 that encodes H60 and Rae1. Like Rae1 proteins, ULBPs are GPI-linked proteins. Therefore, although the ULBPs are only distantly related in sequence to Rae1 and H60 (22–24% amino acid identity), it is tempting to speculate that Rae1, H60 and ULBP define a related family of ancient MHC class I-like gene products that function as ligands for various effector cells in the innate and adaptive immune systems.

## Biological function of the NKG2D receptor–ligand system

### NK-cell activation

Expression of MIC, Rae1, H60 or ULBPs on target cells led to a strong induction of NK cytotoxicity (33, 78–80). Interestingly, activation of human NK cells by cell lines transfected with NKG2D ligands overrode inhibitory signaling mediated by target cell MHC class I molecules (78, 80). It remains to be determined whether activation through NKG2D is completely refractory to inhibitory signaling or alternatively represents a strong stimulatory signal that simply shifts the balance of signaling sharply in favor of NK-cell activation. In addition to stimulating NK-cell cytotoxicity, ectopic expression of Rae1 or H60 by stimulator cells led to a potent NKG2D-dependent stimulation of IFN-$\gamma$ release from mouse NK cells (33). Furthermore, triggering of NKG2D by immobilized ULBP2 led to a potent induction of GM-CSF, TNF-$\beta$ and I-309 (80), the latter a CC chemokine. In the latter experiments, there was a strong synergistic effect with IL-12 (80). The role of NKG2D in cytokine production requires further clarification, in light of a report that immobilized anti-NKG2D failed to induce GM-CSF or IFN-$\gamma$ production from a human polyclonal NK-cell line (86).

### Macrophages

As discussed above, stimulation of macrophages (MØ) and MØ-cell lines with LPS led to a strong induction of NKG2D mRNA and protein ((33) and our unpublished data). Co-stimulation of LPS-activated MØ with stimulator cells ectopically expressing Rae1 or H60 led to a synergistic increase of nitric oxide and TNF-$\alpha$ production from MØ (33). These findings suggest that NKG2D-mediated activation of MØ, in addition to NK cells, may play a role in the innate immune response.

### CD8$^+$ T cells

Virtually all human CD8$^+$ T cells express NKG2D (78). Groh et al. recently reported that NKG2D is a very potent co-stimulatory receptor on human CD8$^+$ T cells (92). Human fibroblasts infected with CMV strongly upregulated MIC expression, whereas MHC class Ia expression was significantly downregulated. CMV-specific CD28$^-$CD8$^+$ T-cell clones exhibited stronger killing of CMV peptide-pulsed target cells if the target cells were transfected with MICA, but did not kill the transfected target cells in the absence of CMV peptide. In assays of cytokine production by these CD8$^+$ T cells, there was also a strong synergistic effect when T-cell receptors and NKG2D were simultaneously stimulated. The co-stimulation was particularly marked for IL-2 production (92), which was not detectably produced in the absence of NKG2D-mediated co-stimulation (92, 101). Thus, the NKG2D receptor provides a potent co-stimulatory signal for human CD28$^-$CD8$^+$ $\alpha\beta$ T cells.

## A fourth strategy of immune recognition

Due to the paucity of information concerning ligands for the stimulatory NK-cell receptors, the "textbook view" in recent years has been that NK cells are regulated primarily by inhibitory receptors. In this conception, NK cells are "tonically" activated by ligands expressed by normal cells, requiring continual inhibition to prevent them from mediating immunopathology. Indeed, it is clear that certain normal (i.e. uninfected, untransformed) class I-deficient cells are sensitive to NK-cell attack, suggesting that certain normal cell types, at least, express ligands capable of activating NK cells as long as inhibitory signals are absent (102–105). However, since not all normal cells are susceptible to NK-cell attack, and many tumor cells are more highly sensitive to NK cells than are normal cells, it was proposed that regulated expression of stimulatory ligands might serve a key role in determining the outcome of the NK cell target–cell interaction (105–107). The

lessons learned from the NKG2D receptor–ligand system provide strong support for the notion that regulated expression of stimulatory ligands (e.g. MIC and Rae proteins) plays a central role in regulating NK-cell activity. Furthermore, the findings that NKG2D expression is upregulated in MØ, $CD8^+$ T cells and possibly $\gamma\delta$ T cells by cellular activation suggests that control of receptor expression can also serve as a critical regulatory step in ensuing immune reactions.

The present data suggest that normal cells are equipped with the capacity to detect "abuse", including infection, transformation and stress, and respond by upregulating ligands for NKG2D such as MIC and Rae1 and probably other ligands that bind to distinct immune receptors. This type of specificity can be viewed as the most recently appreciated of at least four strategies of immune recognition. One of the oldest strategies in evolutionary terms is exemplified by the Toll receptor system. In this form of recognition, immune receptors specific for structures associated with microbes, including bacterial cell wall constituents, bacterial flagella and bacterial DNA, stimulate an important innate response by MØ and other cells ((108–112) and for review (113)). This form of recognition has been described as being specific for "molecular patterns" associated with pathogens (114, 115). A second strategy is represented by the adaptive immune system, which is endowed with the capacity to respond to essentially any non-self molecular entity by virtue of an enormous molecular diversity of receptor sequences. Adaptive immunity is clearly a relatively recent event in evolutionary terms. A third strategy to be considered is exemplified by the capacity of NK cells to recognize missing self, that is, to attack cells that downregulate MHC expression (116). This strategy can be viewed as a complementary strategy of the adaptive response, since it presumably evolved to protect the host from cells that evade recognition by conventional $CD8^+$ T cells. The implication that missing self recognition is a relatively recent adaptation has always been disturbing, however, because NK cells are usually considered to be relatively ancient cells in evolutionary terms (117). A resolution is suggested by the existence of the fourth class of recognition, represented by the capacity of NK cells and other immune cells to respond to "induced-self" ligands such as Rae1 and MIC, expression of which is regulated by various forms of cellular abuse. It is attractive to suggest that the latter form of recognition evolved first, and represents the original principle of recognition by primordial NK cells. The inhibitory receptor system may have been evolved later as a complementary scheme to further regulate NK-cell activity.

It is evident that induced-self antigens can be of sufficient strength, at least in the case of the NKG2D receptor-ligand system, to override inhibitory signals provided by inhibitory receptors specific for MHC class I molecules (78, 80) (Diefenbach & Raulet, unpublished data). Indeed, as mentioned above, further experiments will be necessary to determine the extent to which activation through the NKG2D receptor can be modulated by inhibitory signals. Nevertheless, with the NKG2D precedent in mind, it is attractive to suggest that the inhibitory and stimulatory receptor systems expressed by NK cells can operate with some independence. Thus, a target cell could become susceptible to NK-cell attack by upregulating Rae1 while maintaining normal class I expression, or by downregulating class I while maintaining "normal" expression of ligands that stimulate NK cells. Some tumor cells are expected to undergo both alterations, downregulating class I while at the same time upregulating expression of stimulatory ligands. Such cells are likely to become especially sensitive to NK-cell attack. A likely example is the YAC-1 tumor cell line, which is especially sensitive to NK cells and is known to express abnormally low levels of class I, as well as to express substantial levels of Rae1 (33, 118) (Diefenbach & Raulet, unpublished data).

Important directions for future studies will include the analysis of mechanisms that regulate expression of the induced-self ligands in tumor cells and infected cells. Also of great interest is the nature of the ligands for other stimulatory receptors (i.e. NKp30, NKp44, NKp46, NKR-P1s) and their regulation by transformation and infection. In addition, a greater understanding of the signaling pathways responsible for activation and inhibition and how they interact will be necessary to fully understand NK-cell recognition.

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