ROLES OF THE NKG2D IMMUNORECEPTOR AND ITS LIGANDS

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According to present concepts, innate immunity is regulated by receptors that determine danger levels by responding to molecules that are associated with infection or cellular distress. NKG2D is, perhaps, the best characterized receptor that is associated with responses to cellular distress, defined as transformation, infection or cell stress. This review summarizes recent findings that concern NKG2D, its ligands, its signalling properties and its role in disease, and provides a framework for considering how the induction of immune responses can be regulated by cellular responses to injury.

NKG2D was first identified in a screen for genes that are expressed preferentially by human natural killer (NK) cells[1,2]. NKG2A, NKG2C and NKG2E complementary DNAs were isolated in the same screen. The corresponding receptors were all type-2 transmembrane receptors with sequence similarities to C-type lectins. Although originally given a common name, subsequent analysis showed that NKG2D should be considered as a distinct receptor. NKG2A, NKG2C and NKG2E proteins are all highly related in sequence, are present as heterodimers with another protein (CD94) and recognize a non-classical MHC class I molecule known as HLA-E (in humans) or Qa1 (in mice)[3]. NKG2D, by contrast, differs markedly in sequence[2], is present as a homodimeric receptor and recognizes one of several cell-surface molecules that are only distantly related to MHC class I molecules (FIG. 1). Nevertheless, the *NKG2D* gene is located next to the other *NKG2* genes in the NK gene complex (NKC)[4] (FIG. 1).

Interest in NKG2D was prompted in part by an interest in identifying the stimulatory receptors that NK cells use to recognize target cells. The NKG2D protein sequence contains a charged amino-acid residue in its transmembrane domain, characteristic of stimulatory immunoreceptors. Subsequent studies have led to a wealth of information concerning the immunological role of NKG2D. As described later, several distinct ligands have been identified, most of which are upregulated by infected, transformed and/or stressed cells. These findings indicated a rationale for the function of the NKG2D–ligand interaction, enabling immune cells to detect various pathological alterations in autologous cells.

The expression pattern of NKG2D

NKG2D transcripts were initially detected in NK cells and some T cells[5]. Subsequent analysis showed expression of NKG2D in various lymphoid and myeloid cell types (TABLE 1). In mice, NKG2D is expressed on the cell surface of almost all NK cells[6]. Mouse $CD8^+$ T cells lack NKG2D expression when naive, but expression of the receptor is induced by antigen-specific T cells during immune responses to viruses, intracellular bacteria and presumably other antigens[6]. Engagement of the T-cell receptor (TCR) is sufficient to induce the expression of NKG2D by cultured mouse $CD8^+$ T cells. NKG2D expression is maintained by mouse antigen-specific $CD8^+$ memory T cells *in vivo*[6]. In contrast to $CD8^+$ T cells, NKG2D is not expressed by conventional peripheral $CD4^+$ T cells in mice, even after activation in cell culture[6]. Approximately 25% of mouse splenic γδ T cells, all of which also express CD44, express NKG2D. Almost all of the γδ T cells in the mouse epidermis (dendritic epidermal T cells, DETCs) also express NKG2D, whereas

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most mouse intestinal intraepithelial γδ T cells do not[6]. A large fraction of NK1.1$^+$ T cells also express NKG2D[6]. Finally, NKG2D is expressed by mouse peritoneal exudate macrophages that are activated with lipopolysaccharide (LPS), interferon-α (IFN-α) mixed with IFN-β, or IFN-γ[6,7].

Most human NK cells also express NKG2D, and the levels are increased by exposure to interleukin-15 (IL-15)[8]. The pattern of receptor expression in humans differs from that in mice in some other cell lineages (TABLE 1). Although resting mouse CD8$^+$ T cells do not express NKG2D, almost all human peripheral blood CD8$^+$ T cells express the receptor before activation, including those that lack expression of the co-stimulatory molecule CD28 (REFS 9,10). Furthermore, essentially all peripheral blood γδ T cells express NKG2D[9], in contrast to the more selective expression by mouse γδ T cells[6]. In addition, although intestinal intraepithelial γδ T cells lack NKG2D expression in mice, almost all human intestinal intraepithelial γδ T cells express NKG2D at low levels, which can be increased by incubation with IL-15 (REF. 11). Also unlike the situation in mice, expression of NKG2D was not detected on LPS-activated human macrophages[12]. Finally, although human CD4$^+$ T cells, similar to mouse CD4$^+$ T cells, do not normally express NKG2D[9], upregulation of expression was observed in a fraction of CD4$^+$ T cells from patients with rheumatoid arthritis[13].

Surprising diversity of NKG2D ligands

MICA and MICB. The first evidence for a protein that binds to NKG2D came from a study showing that a soluble form of MHC class-I-chain-related protein A (MICA) — a non-classical class I molecule that is encoded in the human MHC — binds to various lymphocyte subsets. A monoclonal antibody was prepared that blocked the interaction, and the antibody was subsequently shown to bind NKG2D[9]. Further analysis showed that MICB — a close relative of MICA — also binds NKG2D. No functional homologues of MICA or MICB have been identified in the mouse MHC, although two mouse genes with some similarities were recently identified on mouse chromosome 7, which is close to the leukocyte-receptor gene complex[14].

Mouse Rae1, H60 and Mult1. Two research groups used soluble forms of mouse NKG2D as staining reagents to detect ligands expressed by mouse cells. Expression of putative ligands expressed by various mouse tumour-cell lines was observed, and cDNAs encoding the ligands were subsequently cloned[7,15]. Surprisingly, two distinct but related proteins, neither similar to MICA nor MICB, were identified as ligands. One of the proteins was retinoic acid early transcript 1 (Rae1), a lipid-linked membrane protein, which had previously been cloned based on its rapid induction of expression by F9 embryocarcinoma cells after treatment with retinoic acid[16]. Rae1β is a member of a subfamily of several closely related (>98% amino-acid identity) proteins, which also show allelic diversity[17,18]. The genes and/or alleles identified so far are known as Rae1α–Rae1ε. The other ligand identified was histocompatibility 60 (H60), a type-1 transmembrane protein, which had previously been cloned based on its role as a minor histocompatibility antigen[19]. Mouse UL16-binding protein-like transcript 1 (Mult1) — a third relative of this growing protein family — was identified more recently and shown to bind NKG2D[20,21]. In pairwise combinations, Rae1, H60 and Mult1 are only 20–28% identical in their amino-acid sequences[21] (FIG. 2).

ULBP or human RAET1. A distinct approach identified three related human proteins that bind to the UL16 protein of human cytomegalovirus (HCMV) and are encoded by a gene family that is homologous to the Rae1/H60/Mult1 gene family[22]. UL16 was studied because it was a candidate virus protein that was involved in evasion of the immune response. The proteins bound by UL16 were designated UL16-binding proteins (ULBPs). Other researchers named the same molecules human RAET1 proteins, in recognition of their relatedness to the mouse proteins, and identified new members, increasing the gene family to at least six functional genes and two pseudogenes[23–25]. Most of the RAET1 proteins, similar to the mouse Rae1 proteins, are attached to the plasma membrane with a lipid linkage and have been shown to bind human NKG2D. However, two family members, RAET1E (ULBP4) and RAET1G, have transmembrane domains[23,26]. Similar to their mouse counterparts, human RAET1 proteins have considerable sequence diversity, ranging as low as 35% amino-acid identity in pairwise comparisons[23] (FIG. 2).

Ligand genes and domain structure. The mouse Rae1/H60/Mult1 and human RAET1 proteins are relatively distinct in sequence (FIG. 2), but all have a similar and unique domain structure, consisting of two ectodomains that are

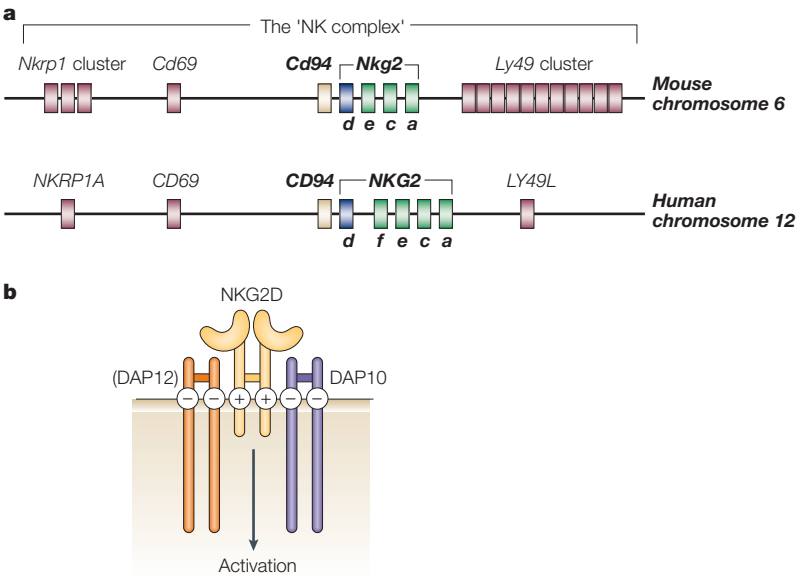

Figure 1 | **The *NKG2D* gene and the NKG2D receptor. a** | NKG2D is encoded in the natural killer (NK) gene complex (NKC). The neighbouring *NKG2* genes, despite their names, are dissimilar in sequence, specificity and function. **b** | The NKG2D receptor is a type-2 transmembrane homodimer that contains charged residues in the transmembrane segments, associates with DNAX-activating protein of 10 kDa (DAP10) and DAP12 signalling molecules, and provides activating signals to lymphocytes.

Table 1 | **Expression of NKG2D by immune cells**

Cell type	Cell-surface expression pattern	
	Mouse	**Human**
NK cells	~100%	~100%
$CD8^+$ αβ T cells	Before activation: absent After activation: ~100% Antigen-specific memory cells:~100%	Before activation: ~100% After activation: ~100% Antigen-specific memory cells: ~100%
$CD4^+$ αβ T cells (conventional)	Rare or absent	Normally absent, upregulated in rheumatoid arthritis patients
$NK1.1^+$ T cells	~70% positive	N. D.
γδ T cells	Splenic γδ T cells: ~25% Intestinal intraepithelial γδ cells: absent Dendritic epidermal γδ T cells: ~100%	Peripheral blood γδ T cells: ~100% Intestinal intraepithelial γδ cells: ~100%
Macrophages	Before activation: absent After activation (with LPS, IFN-α/β or IFN-γ): ~100%	Absent on monocytes and macrophages

IFN, interferon; LPS lipopolysaccharide; N. D. not determined; NK, natural killer.

distantly related to the α1 and α2 domains of MHC class I molecules. The corresponding mouse and human genes are clustered in syntenic regions of the mouse and human genomes — on chromosome 10 in mice[17,19] and the long arm of chromosome 6 in humans[23]. This family of functionally related genes therefore seems to be a newly appreciated gene complex. By contrast, MICA and MICB in humans, although also acting as ligands for NKG2D, are encoded by genes in the MHC gene complex, and also differ in that they contain an α3-like domain[27,28]. Despite the presence of the α3-like domain, MICA and MICB do not associate with $\beta_2$microglobulin[28].

Ligand binding and structure. The binding of NKG2D to its various ligands is generally of higher affinity than many immunoreceptor–ligand interactions. The dissociation binding constants range from ~1 x 10^{-6} M to 4 x 10^{-9} M (REFS 20,29–31) (FIG. 3).

Three-dimensional structure analyses indicate that MICA, Rae1β and ULBP3 are similar, in that the α1 and α2 domains resemble the MHC-like domain, except that the site corresponding to the MHC peptide-binding groove is closed off, apparently preventing the binding of peptides and other small molecules[32–34]. The structures of all three of these ligands in complex with the corresponding human or mouse NKG2D receptor[35] showed that the receptor binds diagonally across the α-helical surface of each of these ligands, similar to the mode of binding of a TCR to an MHC molecule[29,33,34]. Notably, most of the receptor amino-acid residues that dominate binding to the different ligands are the same, and several of the contact residues on the ligands are conserved, especially those that are thought to contribute most of the binding energy[36]. Therefore, despite the marked differences in their amino-acid sequences, the different ligands interact with NKG2D similarly, and the receptor does not seem to undergo marked conformational changes (induced fit) to accommodate different ligands[36]. Another interesting feature of the structures is that different residues in the two NKG2D monomers that make up the dimer dominate binding to the asymmetric α1 and α2 domains of the ligands[32–34,36].

Expression patterns of NKG2D ligands

The various NKG2D ligands have distinct patterns of expression, indicating that they cannot be considered simply redundant in function. Although much remains to be learned about the patterns of expression of the different ligands, a theme is that their expression by normal cells in adults is generally absent or present at low levels, but in pathological conditions their expression is often upregulated. This theme is most clearly exemplified by MICA or MICB in humans, and Rae1 and in some cases H60 in mice. In normal humans, MICA or MICB are expressed only by intestinal epithelial cells, perhaps as a consequence of stimulation by the neighbouring bacterial flora[28]. Expression of MICA and MICB is upregulated by many tumour-cell lines and primary tumours of epithelial origin[37,38]. Upregulation of MICA or MICB expression by these cells is thought to result from the activation of heat-shock transcription elements in the promoters of the corresponding genes — an event known to accompany transformation[28]. Expression of MICA and MICB by dendritic cells can also be induced by IFN-α[39].

Similar to MICA or MICB molecules, Rae1 molecules are not expressed by most normal cells in adult mice[17]. Developmentally, Rae1-encoding messenger RNA, especially the Rae1β and Rae1γ isoforms, is expressed diffusely throughout early embryos, especially in the brain[17,18]. By 18 days of gestation, however, expression of the transcripts is downregulated and remains so in all of the normal adult tissues that have been examined[18]. Similar to MICA and MICB, however, expression of Rae1 molecules is markedly upregulated by various tumour-cell lines[7,15]. Upregulation of expression of H60 and Rae1 transcripts was also observed in skin tissue that was treated with carcinogens and in the resulting carcinomas[40].

In contrast to Rae1 and MICA or MICB, some of the RAETI or ULBP molecules in humans, and Mult1 in mice, are expressed at marked levels by various normal cells at the mRNA level[22,23], but cell-surface expression by normal cells is low or has not been documented. For example, C57BL/6 thymocytes contain high levels of

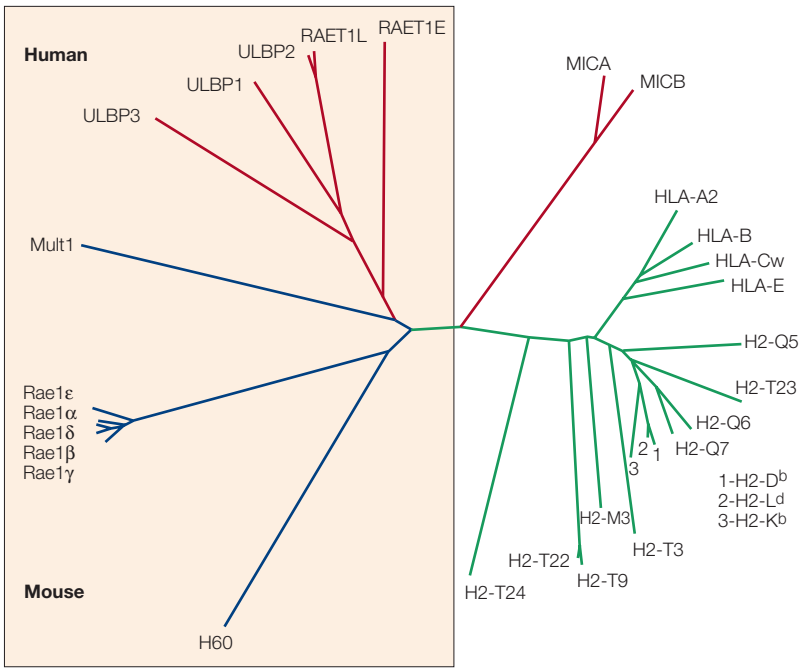

Figure 2 | **The diverse nature of NKG2D ligands.** A dendrogram representing the relatedness of different NKG2D ligands to each other and to various MHC class I molecules. The extent of the sequence relatedness between two proteins is shown by the total distance of the lines that connect those proteins. As a guide, retinoic acid early transcript 1 (Rae1) and H60 proteins are 20–28% identical at the amino-acid level. The NKG2D ligands are shown in blue (mouse) or red (human). The box groups the larger family consisting of human and mouse Rae1, H60, Mult1, ULBP and RAET1 proteins, to distinguish them from the MHC class-I-chain-related protein A (MICA) and MICB ligands. Figure reproduced with permission from REF. 21 © Wiley-VCH (2003). Mult1, mouse UL16-binding protein-like transcript 1; ULBP, UL16-binding protein.

Mult1 mRNA, but stain poorly with NKG2D tetramers[21]. However, ULBPs and probably Mult1 are expressed at functional levels on the cell surface of numerous tumour-cell lines, indicating that these molecules might be regulated at a level other than transcription[21,41]. At present, H60 is the only ligand that is known to be expressed at high levels by normal adult cells, in particular thymocytes in BALB/c (not C57BL/6) mice[42]. It is probable, however, that the thymus is inaccessible to most mature lymphocytes that are potentially responsive to H60, which might help to explain the failure of these mice to develop H60-induced NK-cell-mediated autoimmunity or tolerance.

Unlike MICA or MICB, heat-shock elements have not been implicated in regulating the expression of Rae1, H60, Mult1 or ULBPs. The expression of Rae1 is upregulated in the F9 embryocarcinoma cell line by retinoic acid[16], but regulation of *Rae1* genes by retinoic acid in other cells has not been documented. In general, cell proliferation by itself is insufficient for induction of the ligands, as indicated by the fact that late-stage mouse embryos have low levels of Rae1 expression[18] and normal proliferating cells in culture usually do not upregulate the expression of ligands to marked levels[7]. So, the signalling events that are responsible for the upregulation of Rae1 or H60 expression by tumour cells are not known.

The expression of NKG2D ligands is also upregulated by cells that are infected with pathogens. The induction of MICA, MICB or ULBP expression by cells infected with HCMV has been observed in cultures of primary fibroblasts and endothelial cells, and in patient samples[10,43]. MICA expression was also upregulated by cells as a result of the binding of *Escherichia coli* adhesin AfaE to cellular CD55 (REF. 44) and by cells infected with *Mycobacterium tuberculosis*[45]. Mouse fibroblasts infected with mouse CMV (MCMV) upregulated the expression of Rae1, but not H60, transcripts[46]. Evidence for the role of NKG2D and its ligands in protection from infection with CMV is discussed later.

The upregulation of expression of NKG2D ligands by tumour cells or infected cells indicates that the system is 'wired' to respond to signalling events or combinations of signalling events that occur in distressed cells, but not normal cells. In this system, apparently the cell itself must recognize that it is undergoing pathological changes and respond by expressing molecules that alert the immune system. In this respect, together with the fact that NKG2D ligands are self molecules, the logic of the system seems to differ fundamentally from that proposed for Toll-like receptors (TLRs), which generally recognize foreign molecular patterns[47] (FIG. 4).

Stimulation of immune cells through NKG2D

NKG2D functions as a stimulatory receptor in various cell types. So far, there is no evidence that the different ligands induce qualitatively distinct biological effects in responding cells, though this remains a clear possibility. Minimally, the various ligands would be predicted to differ quantitatively in their effects based on the marked differences in their affinity for NKG2D (FIG. 3). At present, the relevance of such differences has not been documented. The consequences of NKG2D stimulation in various NKG2D-expressing cell types are described below.

Natural killer cells. Tumour-cell lines transfected with NKG2D ligands have enhanced sensitivity to lysis by NK cells[7,9,15,22]. In general, the lysis of tumour cells that naturally express NKG2D ligands is partially inhibited by NKG2D-specific antibodies, indicating that NKG2D is an important receptor in the recognition of target cells by NK cells, but not the only one[6,41]. Indeed, some target cells that lack expression of NKG2D ligands are nevertheless sensitive to NK cells[6], in line with the identification of other NK-cell stimulatory receptors that participate in tumour-cell recognition[48].

Antibody crosslinking of NKG2D expressed by IL-2- or polyI:C-activated mouse NK cells triggers calcium mobilization and IFN-γ production[6]. In human NK cells, crosslinking with multivalent soluble ligands (ULBPs) stimulates the production of several cytokines including IFN-γ, tumour-necrosis factor (TNF), lymphotoxin and granulocyte–macrophage colony-stimulating factor (GM-CSF), as well as chemokines such as CCL4 (macrophage inflammatory protein 1β, MIP1β) and CCL1 (I-309)[8,22,49]. Limiting concentrations of IL-12 act synergistically with ULBPs to trigger the production of cytokines by human NK cells[8,22,49]. Unlike multivalent

IMMUNORECEPTOR TYROSINE-BASED ACTIVATION MOTIF (ITAM). A short sequence found in the cytoplasmic domains of numerous immune receptors. After receptor engagement, tyrosines in the ITAM are phosphorylated, enabling the recruitment and activation of SYK or ZAP70 protein tyrosine kinases.

ULBPs, multivalent NKG2D-specific antibody failed to stimulate the production of IFN-γ by human NK cells[50]. The differing results depending on the crosslinking reagent that was used might result from differences in reagent affinity, the participation of other receptors, the activation state of the cells used or qualitative differences in the crosslinking[51]. Although plate-bound NKG2D-specific antibody failed to induce IFN-γ production, it did stimulate the release of granzymes that participate in the cytotoxicity reaction[50].

Interestingly, expression of NKG2D ligands by target cells that express normal levels of MHC class I molecules can, in some cases, result in marked lysis by NK cells[7,9,12,15,22]. Although it was initially proposed that stimulation of cells by NKG2D is refractory to inhibitory signals that are mediated by MHC-specific receptors, it seems instead that the NKG2D signal is strong enough to overcome inhibitory signalling by MHC-specific receptors in some cases[12].

Macrophages. The consequences of NKG2D engagement by macrophages were investigated with peritoneal exudate macrophages that had been pretreated with limiting doses of LPS or with IFN-α/β to stimulate the upregulation of receptor expression. When co-cultured with cell lines that were transfected with NKG2D ligands, the macrophages were induced to secrete nitric oxide and TNF[7,21].

T cells. Activation of $CD8^+$ T cells by MICA or MICB was investigated in the case of HCMV-specific T cells in immune individuals. When pre-incubated with the appropriate HCMV-derived T-cell epitope, transfected target-cell lines expressing MICA or MICB were more effective than parental cell lines at stimulating T-cell activation and the production of IFN-γ, TNF, IL-2 and IL-4 (REF. 10). In mice, expression of Rae1 or H60 by target cells resulted in enhanced cytolysis and the production of IFN-γ by cytotoxic T lymphocytes (CTLs) specific for tumour antigens[52]. Notably, it was shown that NKG2D is expressed by human $CD8^+$ T cells that lack expression of the CD28 co-stimulatory molecule. The $CD8^+CD28^-$ subset is a memory T-cell population that comprises a large fraction of $CD8^+$ T cells in normal adult humans, especially in older humans, and which was previously considered to be unresponsive to antigen. The production of IL-2 by HCMV-specific T cells of this phenotype occurred only when the antigen-presenting cells expressed MICA[10].

NKG2D ligands also have a role in the activation of certain γδ T-cell subsets. The activation of Vδ1-expressing human γδ T-cell clones obtained by stimulating T cells extracted from intestinal epithelial tumours with MICA-positive or MICB-positive stimulator cells depends on the expression of MIC by the target cells[53]. On these clones, but not other γδ T-cell clones, MIC can bind to both NKG2D and the γδ TCR and is a sufficient stimulus for activation of the cells[9,22]. Vδ1-expressing cells are common in certain tissues, but the proportion with TCRs that are specific for MIC in normal humans has not been determined directly. Interestingly, Vδ1-expressing T cells are often clonally expanded in human transplant patients that are infected with HCMV[54] — a virus that is known to cause the upregulation of MIC expression by infected cells. NKG2D is also expressed by a dominant population of γδ T cells in peripheral blood that expresses the Vγ9/Vδ2 TCR. Probably because this TCR is specific for small organic phosphate molecules and not MIC, stimulation by MIC is not sufficient to activate these cells, but does provide an enhancing or co-stimulatory signal for activation[45]. In mice, cytolysis of sensitive tumour-cell lines by γδ TCR-positive DETC lines was partially inhibited by blockade of NKG2D[40]. It is probable that NKG2D also has a role in the activation of other γδ T-cell subsets.

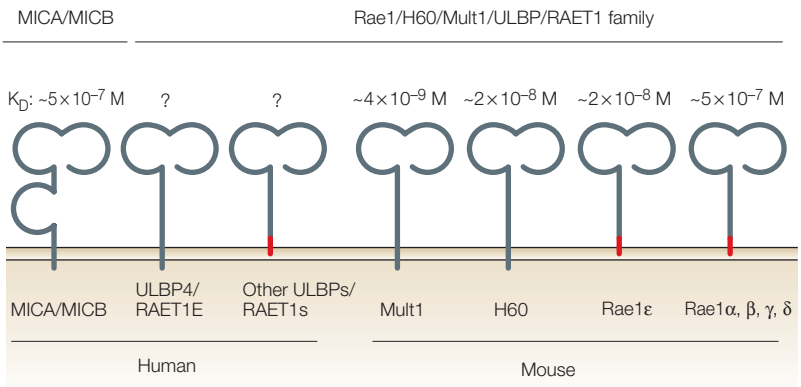

Figure 3 | **Domain structures and affinities of various NKG2D ligands.** MHC class-I-chain-related protein A (MICA) and MICB, unlike the other ligands, contain an α3-like domain. Despite the presence of an α3-like domain, they fail to bind β_2-microglobulin — the light chain of MHC class I molecules. Some of the ligands, such as retinoic acid early transcript 1 (Rae1) and some UL16-binding proteins (ULBPs) are linked to the membrane by glycosylphosphatidylinositol (GPI) anchors (shown in red), whereas others, such as MICA and MICB, H60, mouse ULBP-like transcript 1 (Mult1) and some ULBPs are type-1 transmembrane proteins. The affinities of the various ligands for NKG2D, represented as the equilibrium dissociation constant (K_D), are shown above each ligand.

Signalling pathways activated by NKG2D

Many primary recognition receptors in the immune system are multichain molecules that consist of subunits responsible for ligand recognition that are non-covalently associated with signalling ('adaptor') subunits. The recognition subunits generally contain a charged residue in the transmembrane domain that enables interactions with oppositely charged residues in the transmembrane segments of the adaptor molecules. Most of these adaptor subunits, including CD3 molecules or DNAX-activating protein of 12 kDa (DAP12), contain a cytoplasmic IMMUNORECEPTOR TYROSINE-BASED ACTIVATION MOTIF (ITAM). Receptor engagement induces tyrosine phosphorylation of the ITAMs, recruitment and activation of SYK or ζ-chain-associated protein 70 kDa (ZAP70) tyrosine kinases, and phosphorylation of downstream effectors that trigger cell activation.

The presence of a charged amino-acid residue in the cytoplasmic domain initially implicated a stimulatory role for NKG2D. Interestingly, the receptor was shown to associate with a new adaptor molecule known as DAP10 (REF. 55), which is unusual as it lacks an ITAM in its cytoplasmic domain and instead contains a different tyrosine-based motif that is similar to

those found in co-stimulatory receptors such as CD28, inducible co-stimulatory molecule (ICOS) and CD19 (REFS 55,56). The latter receptors amplify activation signals that are provided by primary recognition receptors. Engagement of NKG2D in human NK cells results in tyrosine phosphorylation of DAP10 and recruitment and activation of the p85 subunit of phosphatidylinositol 3-kinase (PI3K) and the anti-apoptotic kinase AKT[8,55], as also occurs downstream of CD28 and ICOS. These findings indicated that NKG2D might function as a co-stimulatory receptor rather than as a primary recognition receptor. Studies of $CD8^+$ T-cell activation stimulated by NKG2D-ligand-transfected target cells or by antibody crosslinking supported the conclusion that NKG2D functions as a co-stimulatory receptor that amplifies TCR-mediated activation of $CD8^+$ T cells[10].

In contrast to the results with T cells, however, crosslinking of NKG2D on mouse NK cells or macrophages with antibodies[6] or on human NK cells with recombinant ligands[8,49] was sufficient to trigger cytokine release from NK cells, and TNF or nitric oxide production by macrophages. In these cells, therefore, NKG2D seems to function as a primary recognition receptor.

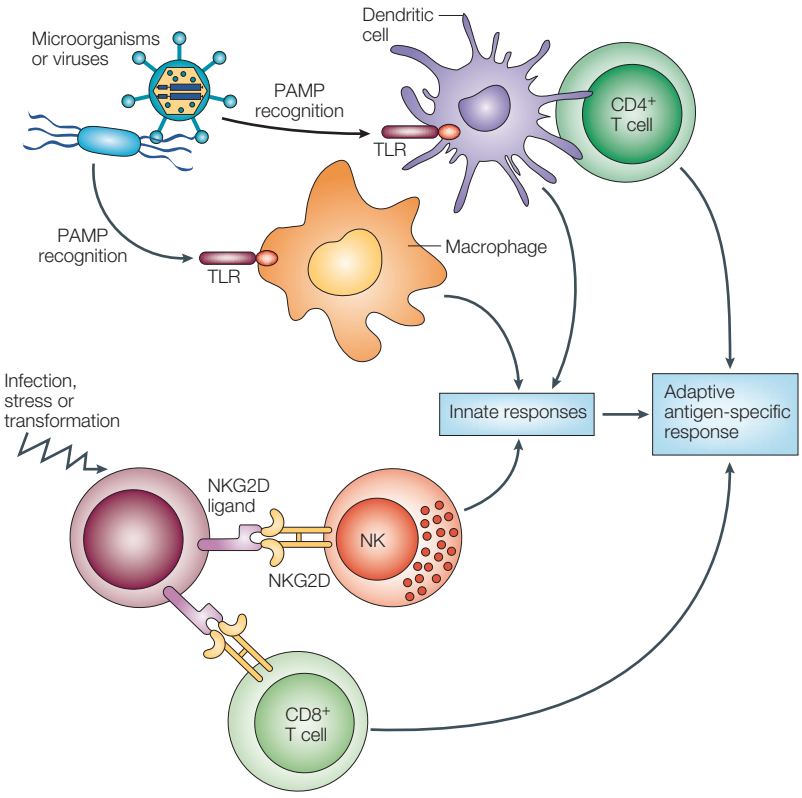

Figure 4 | **Comparative roles of Toll-like receptors and NKG2D in immune recognition.** Both types of receptor activate innate immunity and amplify adaptive immune responses. Whereas most Toll-like receptors (TLRs) recognize pathogen-associated molecular patterns (PAMPs), such as bacterial cell-wall structures or viral RNA intermediates, NKG2D recognizes autologous ligands that are upregulated by transformation, infection or cell stress. The distinctiveness of these two innate recognition systems might ultimately blur, however, as it is possible that TLR signalling participates in upregulating the expression of NKG2D ligands or that the two receptor systems signal synergistically. Furthermore, TLRs specific for self molecules have recently been reported[75,76]. NK, natural killer.

Distinct functions of NKG2D in different cell types is due in part to the existence of two NKG2D protein species encoded by distinct mRNA splice isoform, and the differential expression of adaptor proteins[57]. The NKG2D-long (NKG2DL) transcript encodes a protein that associates with DAP10 but not DAP12. By contrast, the product of the NKG2D-short (NKG2DS) transcript is 13 amino acids shorter in the cytoplasmic domain and associates with both DAP10 and DAP12. As discussed earlier, DAP12, unlike DAP10, contains a cytoplasmic ITAM. Both NKG2D transcripts are present in activated NK cells, $CD8^+$ T cells and macrophages, but T cells, unlike NK cells and macrophages, do not express DAP12 (FIG. 5). Immunoprecipitation experiments confirmed that activated $CD8^+$ T cells contain NKG2D–DAP10 complexes, whereas NK cells and macrophages contain both NKG2D–DAP10 and NKG2D–DAP12 complexes[57,58]. Furthermore, NK cells and macrophages from mutant mice that lack functional DAP12 signalling were defective in NKG2D-induced cytokine production, and the mutant NK cells had reduced capacity to kill target cells that expressed NKG2D ligands, with lysis of some target cells being more affected than others[57]. Conversely, the inability of NKG2D crosslinking by itself to induce cytokine production by $CD8^+$ T cells was reversed when DAP12 was expressed by these cells from a transgene[57]. Crucial independent evidence that signalling molecules, other than DAP10, have a role in NKG2D triggering in mouse NK cells came from the demonstration that DAP10-deficient mice have only partially impaired NK-cell responses to target cells that express NKG2D ligands[58].

These findings indicated that the differential association of NKG2D with signalling adaptor molecules confers the receptor with functions that are appropriate to the particular cell type. In NK cells and macrophages, the receptor provides signals that lead to full activation of the cells, whereas in T cells the receptor enhances antigen-specific responses that rely on the TCR for primary specificity. The flexibility that allows a single recognition subunit to provide different signals depending on the cells involved, if true for other receptors, might be one evolutionary rationale for the general multisubunit nature of stimulatory immunoreceptors.

The association of NKG2D with DAP12 was observed in mouse NK cells that had previously been activated with double-stranded RNA (dsRNA) *in vivo*, or by culturing the cells for a few days in the presence of IL-2. Interestingly, unactivated NK cells from normal mice lack the NKG2DS isoform, and so presumably lack NKG2D–DAP12 complexes[57] (FIG. 5). This observation might partly account for findings that unactivated NK cells from mice often respond poorly to target cells, whereas NK cells activated *in vivo* with polyI:C or viruses have increased cytotoxicity *in vitro*[52,59,60] and are more effective in eliminating tumours *in vivo*[59,61]. The finding that the DAP12-associated form of NKG2D is induced by an innate stimulus such as dsRNA or by cytokines such as IL-2 indicates that signals induced during infections can convert NK cells to a highly responsive state. Type 1 interferons — the main cytokines induced by

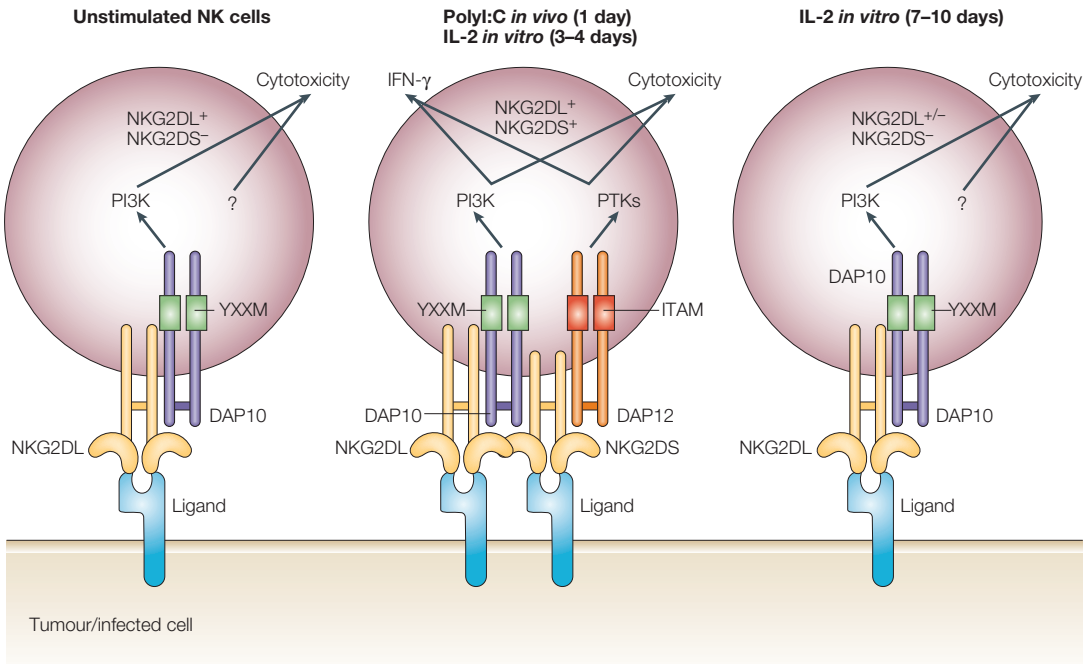

Figure 5 | **Associations of NKG2D with signalling molecules in mouse natural killer cells.** Unstimulated natural killer (NK) cells mainly express the long isoform of NKG2D (NKG2DL), which associates with DNAX-activating protein of 10 kDa (DAP10) and not DAP12 and therefore is predicted to activate the phosphatidylinositol 3-kinase (PI3K)-initiated pathway. NK cells activated with polyI:C *in vivo* or briefly with interleukin-2 (IL-2) in cell culture upregulate the expression of the short isoform of NKG2D (NKG2DS), which associates with both DAP12 and DAP10; the DAP12-associated form activates SYK or ζ-chain-associated protein 70 kDa (ZAP70) protein tyrosine kinases (PTKs), whereas the DAP10-associated form activates PI3K. Engagement of NKG2D provides a signal that is sufficient to induce the production of interferon-γ (IFN-γ) and cytolysis by these cells. Long-term culture in the presence of IL-2 results in the downregulation of expression of both isoforms of NKG2D, especially the short form, indicating that NKG2D in these cells mainly signals through DAP10. Activation of killing and cytokine release occurs when both the DAP10- and DAP12-associated forms are activated. The DAP10-associated form of NKG2D might suffice to activate killing (but not cytokine release) in some circumstances, although other signals might also be required (indicated by question marks). The figure refers to mouse NK cells; in human NK cells, a long-form equivalent of NKG2D associates with DAP10, but a short form equivalent has not been identified. ITAM, immunoreceptor tyrosine-based activation motif.

dsRNA — are also produced in animals with tumours, and genetic evidence indicates that such interferons are protective for the host[62]. So, it is possible that type 1 interferons induced in animals with tumours enhances the antitumour activity of NK cells, in part by converting NKG2D to a more active form. The conversion of NKG2D to the active form might also occur as a result of signals through other NK-cell receptors.

Recent studies confirm that cytokine production by NKG2D-stimulated NK cells depends on DAP12 and associated SYK and/or ZAP70 tyrosine kinases. However, these studies provide a more complex overall picture of NKG2D function as they indicate that the induction of cytolysis, can in different circumstances, be stimulated by both DAP12-dependent and DAP12-independent mechanisms[50,63]. For example, whereas cytolysis of Rae1- or H60-transfected RMA cells by freshly isolated NK cells was dependent on the SYK-family kinases that associate with DAP12, lysis by NK cells that had been cultured for several days in the presence of IL-2 showed less dependence on these kinases[63]. Furthermore, human NK-cell lines that lack the DAP12-associated NKG2D isoform could mediate REDIRECTED KILLING of target cells coated with NKG2D-specific antibody[50]. In the mouse studies, it was not ruled out that signalling molecules, other than DAP10 or DAP12, associate with NKG2D and are required to trigger killing, but in the human studies, redirected killing was induced with an antibody specific for a transfected chimeric molecule consisting of the CD4 extracellular domain attached directly to the DAP10 intracellular domain[50]. In this latter system, DAP10 did not activate SYK, ZAP70 or linker for activation of T cells (LAT), but did associate with several important signalling molecules, including SH2-domain-containing leukocyte protein of 76 kDa (SLP76), VAV1, RHO–RAC family GTPases and phospholipase C-γ2 (PLC-γ2)[50]. Although cytolysis was induced under these conditions of activation, the production of IFN-γ was not, consistent with the mouse studies showing that IFN-γ production is DAP12 dependent.

So, signalling by DAP12 or the associated SYK or ZAP70 kinases is required to trigger NKG2D-dependent cytokine release by NK cells — an important effector function of the cells — but these signalling molecules are not always required to trigger NK-cell-mediated killing. It cannot be concluded on this basis that the NKG2D–DAP10 signal is sufficient to trigger killing, because the target cells used probably present ligands for various other receptors, some of which could act

REDIRECTED KILLING
An experimental system for determining the capacity of a natural killer (NK)-cell receptor to induce cytotoxicity. NK cells coated with antibody specific for a candidate receptor are assessed for their ability to kill target cells that express an Fc receptor to which the antibody binds.

synergistically with DAP10 signalling. In this light, it is interesting that the requirement for DAP12 in cytolysis by mouse NK cells varied for different target cells. For example, lysis of ligand-transduced B16 cells was more dependent on DAP12 than lysis of ligand-transduced RMA cells[57], and lysis of ligand-transduced RMA cells by freshly isolated NK cells was more dependent on SYK-family kinases than was lysis of ligand-transduced Baf3 cells[63]. So, target cells with a lower dependency on DAP12 could express ligands for other receptors that synergize with DAP10 to activate cytolysis.

The association of NKG2D with DAP12 has been observed in mouse NK cells and macrophages, but no association of human NKG2D with DAP12 was observed in human NK cells[50,64], nor has a short isoform of NKG2D been detected in human cells. Human NKG2D might fail to interact with DAP12, although an explanation is required for the finding that crosslinking with soluble ULBPs leads to cytokine production by human NK cells[49]. It remains possible that expression of a short form of human NKG2D can also be upregulated by human NK cells under conditions that remain to be discovered.

Role of NKG2D ligands in tumour-cell surveillance

As shown by transfection experiments and blocking antibodies, natural or induced expression of NKG2D ligands markedly enhances the sensitivity of tumour cells to NK cells *in vitro*[6,7,9,15,41]. The ability of $CD8^+$ T cells or γδ T cells to attack tumour cells is also increased if the tumour cell expresses NKG2D ligands, although in this case the antigen receptor that is expressed by the T cells must also be specific for the tumour[45,52]. In the case of $CD8^+$ T cells, the relevant antigens are presumably tumour antigens that are presented by MHC class I molecules. Some γδ T cells might also be specific for tumour antigens, but in at least one case, as discussed earlier, the antigen receptor is specific for MICA itself[65].

Expression of NKG2D ligands by tumour cells also results in immune destruction *in vivo*. For example, the B16-BL6 melanoma-cell line, which lacks expression of NKG2D ligands, is one of the most tumorigenic and least immunogenic cell lines used for subcutaneous transfer studies of tumour immunity. Relatively high doses of B16-BL6 cells that are transfected with Rae1 or H60 were efficiently and permanently rejected by syngeneic B6 mice[52]. Similar results were obtained with the RMA cell line, which is also highly tumorigenic[52,66], and the EL4 cell line, which is less so[52]. Immune-depletion studies showed that rejection was dependent on NK cells and/or $CD8^+$ T cells depending on the parent tumour-cell line and the dose of tumour cells that were transferred[52]. These studies, together with the *in vitro* studies, leave little doubt that expression of NKG2D ligands confers an effective barrier to tumour formation.

However, the evidence that NKG2D ligand expression by tumour cells is a barrier to tumour growth must be reconciled with the finding that many primary tumours and tumour-cell lines naturally express NKG2D ligands[7,37,41]. It is possible that tumour cells often express insufficient levels of NKG2D ligands to stimulate tumour-cell rejection, either because expression of the ligands is not sufficiently upregulated early in the development of the tumour, or because tumour cells with lower levels of ligand expression are selected by the immune system *in vivo* as the tumour evolves. Direct experimentation showed that less rejection occurred when transfected tumour cells expressed only intermediate levels of Rae1, similar to the levels of expression by many tumour-cell lines[52]. Ligand-expressing tumours might also evolve mechanisms to evade NKG2D-mediated immunity, as indicated by the evidence that human tumours that express MICA often produce a soluble version of MICA that reaches high levels in the serum and causes a systemic desensitization of NKG2D in T cells (and possibly other immune system cells)[67,68]. However, the existence of tumours that evade NKG2D does not detract from the possibility that many other tumours are successfully eliminated by NKG2D-mediated immune activation, because such evasion mechanisms are unlikely to be universally effective.

Notably, mice that had rejected tumour-cell transfectants that expressed NKG2D ligands were resistant to subsequent challenge with the parental tumour cells that lacked NKG2D ligands. Priming with ligand-transfected cells induced immunity against all of the three parental tumour-cell lines that were tested, including the poorly immunogenic and highly aggressive B16-BL6 melanoma-cell line and the highly tumorigenic RMA lymphoma-cell line[52]. The induction of long-lasting immunity by NKG2D ligand-transfected RMA cells was confirmed in two independent studies[21,69], but was not observed in another study[66]. The basis for this discrepancy, which might include differences in the cells or the ligands used, has not been determined.

NKG2D ligand-transfected tumour-cell lines failed to induce long-term immunity in mice that lack $CD8^+$ T cells[52]. Consistent with a role for T cells, immunity induced with a particular tumour-cell line was specific for that cell line. Irradiated tumour-cell transfectants retained their immunogenicity and resulted in priming of $CD8^+$ T cells that were specific for tumour-cell antigens in cases where the irradiated parental tumour-cell line was non-immunogenic[52]. The identity of the tumour antigens that were involved has not yet been determined. The ability of ligand-transfected cells to induce tumour immunity indicates the promise of NKG2D ligands as adjuvants in tumour vaccines.

How NKG2D ligands expressed by tumour cells enhance priming of tumour antigen-specific $CD8^+$ T cells remains to be established. Depletion of NK cells before vaccination failed to prevent T-cell priming, indicating that NK-cell-derived cytokines or the formation of tumour debris as a result of NK-cell-mediated tumour-cell lysis are not required for this process[52], although they might contribute to it. An alternative possibility is that NKG2D ligands expressed by the tumour cells directly co-stimulate tumour-antigen-specific $CD8^+$ T cells[10]. It is also possible that the activation of antigen-presenting cells that express NKG2D is increased by ligand-expressing tumour cells, leading to more effective processing and/or presentation of tumour antigens.

Role of NKG2D ligands in virus infections

As already summarized, infection with CMV leads to the upregulation of expression of NKG2D ligand transcripts, including MICA or MICB and ULBPs in humans, and Rae1 in mice[10,43,46]. Opposing this effect, both HCMV and MCMV encode proteins that interfere with ligand expression at the cell surface[46,70–73]. The HCMV-encoded UL16 protein binds to at least two different ULBPs and MICB. In infected cells, UL16 retains MICB, ULBP1 and ULBP2 in an internal compartment[43,71–73]. In mice, the gp40 protein encoded by the MCMV *m152* gene, previously shown to inhibit the expression of MHC class I molecules, also inhibits the expression of Rae1 and possibly other ligands by MCMV-infected cells[46,70]. Viruses with the mutated *m152* gene show reduced virulence in the early stages of infection that can be reversed by treatment with NKG2D-specific antibody, indicating that gp40 enhances virulence by blocking the expression of Rae1 (REF. 46). These viral evasion mechanisms clearly reduce the expression of NKG2D ligands, but are not completely effective in doing so, as shown by the findings that cells infected with wild-type virus stimulate NK cells and $CD8^+$ T cells in a partially NKG2D-dependent manner[10,46]. It might therefore be probable that blocking NKG2D would lead to reduced virus surveillance by NK cells. However, blocking NKG2D did not lead to increased virus loads in the early phases of infection with wild-type MCMV[46]. So, it seems that MCMV effectively opposes the NKG2D sentinel system *in vivo*, at least for the conditions used and the mouse and virus strains that were tested. The detailed role of NKG2D and its ligands in other infections awaits elucidation.

Concluding remarks

The NKG2D-ligand system seems to have evolved to function as a multi-functional sentinel system to alert the immune system in response to infections and tumours. Stimulation through the receptor can lead to the enhancement of innate immune functions, mediated by NK cells and myeloid cells (and possibly NK1.1-positive T cells), and the enhancement of adaptive immunity, mediated by $CD8^+$ and γδ T cells. The diverse expression patterns of different ligands indicates they might be specialized to respond to distinct indications of cellular distress. Future investigations of ligand regulation might elucidate some of the signalling pathways that cells use for determining different forms of distress. The role of this system in triggering immune responses might also position it in pathways that can lead to aberrant activation of the immune system leading to autoimmunity[13,74] or other forms of immunopathology. Finally, it is probable that the NKG2D system interacts in a dynamic manner with other innate-recognition systems. It will be interesting to determine the extent to which cross-talk between these different innate-recognition systems leads to qualitative differences in immune responses.

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