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Effect of NKG2D ligand expression on host immune responses

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Summary

NKG2D (natural-killer group 2, member D) is an activating receptor present on the surface of natural killer (NK) cells, some NKT cells, CD8⁺ cytotoxic T cells, $\gamma\delta$ T cells, and under certain conditions CD4⁺ T cells. Present in both humans and mice, this highly conserved receptor binds to a surprisingly diverse family of ligands that are distant relatives of MHC-class-I molecules. There is increasing evidence that ligand expression can result in both immune activation (tumor clearance, viral immunity, autoimmunity, and transplantation) and immune silencing (tumor evasion). In this review, we describe this family of NKG2D ligands and the various mechanisms that control their expression in stressed and normal cells. We also discuss the host response to both membrane-bound and secreted NKG2D ligands and summarize the models proposed to explain the consequences of this differential expression.

Keywords

natural killer cell; NKG2D; NK cells; MIC; cytotoxicity; cancer; soluble ligands

Introduction

Cytotoxic lymphocytes include cytotoxic T lymphocytes (CTLs), $\gamma\delta$ T cells, and NK cells. NK cells are a component of the innate immune system and use multiple receptors to recognize their targets. NK cell cytotoxicity is determined by a balance of signals (1) emanating from the recognition of “missing-self” (2) and “induced-self” on target cells. “Missing-self” recognition has been extensively studied and involves the activation of NK cells when they encounter cells with low or absent expression of major histocompatibility complex class I molecules. In this circumstance, the inhibitory receptors for MHC class I, which have immunoreceptor tyrosine-based inhibition motifs (ITIM) in their cytoplasmic domains, are not engaged by MHC ligands on the target cells, thereby allowing an immune response to proceed. Such inhibitory receptors include the human killer cell immunoglobulin-like receptors (KIR), members of the mouse Ly49 receptor family, and the CD94/NKG2A receptor. Their MHC ligands are well characterized and have been reviewed elsewhere (1). In contrast, NK cell recognition of “induced-self”, or self-proteins upregulated in infected or transformed cells, is only partially understood. Although many NK cell activating receptors have been proposed to mediate “induced-self” recognition, the exact ligands recognized remain unknown in many cases. An exception to this is NKG2D, an invariant receptor shared by NK cells and T cells that can potently induce killing. NKG2D recognizes a family of “induced-self” ligands that has been

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extensively studied and remains a field of active investigation. Here, we review the discovery of the NKG2D ligands and their striking diversity in structure, expression patterns, and regulation. In addition, we discuss the host response to both membrane-bound and secreted NKG2D ligands and summarize the models proposed to explain the consequences of this differential expression.

NKG2D receptor complex and signaling

NKG2D is a type II transmembrane-anchored C-type lectin-like receptor expressed as a disulfide-linked homodimer on the cell surface and is encoded by the *KLRK1* (killer cell lectin-like receptor subfamily member 1) gene. *KLRK1* is highly evolutionarily conserved and is located on chromosome 6 in mice and on a syntenic region of human chromosome 12. Essentially all NK cells, most NKT cells, subsets of $\gamma\delta$ T cells, all human CD8⁺ T cells, activated mouse CD8⁺ T cells, and a subset of CD4⁺ T cells express NKG2D. NKG2D-deficient mice have recently been generated. These mice exhibit normal lymphocyte numbers, including NK cells, in all organs analyzed (3). In addition, NKG2D-deficient NK cells express normal levels of maturation markers, as well as an intact repertoire of activating and inhibitory receptors (3), although subtle effects on NK cell development have been reported (4). NKG2D-deficient mice are impaired in the killing of certain tumors *in vitro* and Guerra *et al.* (3) also showed that they had an increased susceptibility to primary tumorigenesis *in vivo*, thus confirming the important role of NKG2D in tumor immunosurveillance. NKG2D has no signaling motif within its short intracellular domain (5), and similarly to many other activating receptors, associates with signal-transducing proteins via charged residues in its transmembrane domain (Fig. 1). In mice, there are two isoforms of NKG2D generated by alternative splicing that differ in the presence or absence of 13 amino acids at the N-terminus in the cytoplasmic domain (6). The long form of NKG2D (NKG2D-L) associates exclusively with the DAP10 adapter protein. In contrast, the short form of NKG2D (NKG2D-S) can pair with either DAP10 or another signal-transducing protein, DAP12 (6,7). This differential adapter pairing has functional consequences, as the different adapters trigger distinct signaling cascades. The cytoplasmic YINM motif of DAP10 recruits the p85 subunit of phosphoinositide kinase-3 (PI3K) and growth factor receptor-bound protein 2 (Grb2) (8,9). DAP12 carries an immunoreceptor tyrosine-based activation motif (ITAM) whose phosphorylation leads to the recruitment of the zeta-chain associated protein kinase 70 (Zap70) and spleen tyrosine kinase (Syk) (10). Thus, NKG2D engagement can result in both the PI3K and Grb2 and Syk and Zap70 signaling cascades. Each NKG2D homodimer associates with two homodimers of DAP10, hence forming a hexameric structure (11). Whether a single NKG2D homodimer can pair with both DAP10 and DAP12 homodimers has not yet been determined, but one could imagine this scenario to be beneficial to induce both signaling cascades upon triggering a single receptor. Crystal structures of both mouse and human NKG2D receptors in the soluble form and bound to ligands have been reported and suggest that NKG2D binds to its ligands through “rigid adaptation” recognition, allowing binding to a wide variety of ligands (12–14).

Discovery of NKG2D ligands

The most remarkable trait of the NKG2D receptor system is the diversity of ligands that can bind to this single invariant receptor. NKG2D ligands are distantly related homologues of MHC class I proteins and new members of this family continue to be discovered in both mice and humans (Fig. 2).

MHC class-I-chain-related protein A (MICA) and B (MICB), which are encoded by genes within the human MHC and are genetically linked to HLA-B, were first described as cell-stress-induced proteins expressed in gastrointestinal epithelium (15). Using a soluble form of MICA, Bauer *et al.* identified its receptor as NKG2D (16). Subsequently, two human cell surface

glycoproteins that bound to the human cytomegalovirus (HCMV) UL16 glycoprotein were described and named UL16-binding protein 1 and 2 (ULBP1 and ULBP2) (17). Similar to MICA and MICB, ULBP1 and ULBP2 also bound to the NKG2D receptor and stimulated human NK cells. Based on sequence homology with ULBP1 and ULBP2, four additional human ULBP family members were described and named ULBP3, ULBP4, RAET1G (or ULBP5), and RAET1L (or ULBP6) (18–20). All six ULBP family members, officially named *RAET1* genes, are encoded in a gene cluster on chromosome 6 (6q24.2–6q25.3), which is syntenic to a region on mouse chromosome 10 that contains the mouse *Raet1* genes which are orthologs of the human *RAET1* genes.

The prototype member of *Raet1* gene family was first discovered as retinoic acid early inducible cDNA clone-1 (Rae-1), which was rapidly induced on F9 teratocarcinoma cells in response to treatment with retinoic acid (21,22). Subsequently, two groups detected binding of a soluble form of mouse NKG2D to mouse transformed cell lines and used expression cloning techniques to identify the NKG2D ligands (23,24), which included Rae-1 and a related protein name histocompatibility antigen 60 (H60) (25). Presently, there are five known members of the Rae-1 family, named Rae-1 α , Rae-1 β , Rae-1 γ , Rae-1 δ , and Rae-1 ϵ , which are differentially expressed in various mouse strains and highly related to each other (>85% identity). The H60 family comprises 3 members. H60a, the first ligand of the family to be described, was initially identified as a minor histocompatibility antigen by immunizing C57BL/6 mice with MHC-identical BALB.B cells (25). Recently, using the amino sequence of H60a as a query, Takeda *et al.* and Whang *et al.* identified two novel members of this family, named H60b and H60c (26,27). Finally, Murine UL-16-binding protein-like transcript 1 (MULT1) is the unique member of the third family of mouse NKG2D ligands and was found by database searching for mouse sequences with similarities to human ULBP (28,29).

Structural nature of membrane-bound ligands

Mouse and human NKG2D ligands are structural homologs of MHC class I molecules but remain a relatively distantly related family. The NKG2D ligands differ widely in sequence, domain structure, and affinity for the NKG2D receptor (Fig. 2). MICA and MICB are encoded within the human MHC, with which they share 28–35% sequence homology. Similarly to MHC class I molecules, MICA and MICB possess three immunoglobulin (Ig)-like domains ($\alpha 1$, $\alpha 2$, and $\alpha 3$) and have a short cytoplasmic tail. Unlike MHC molecules, MICA and MICB do not associate with $\beta 2$ -microglobulin or bind peptides. Indeed, the $\alpha 1$ and $\alpha 2$ domains lack the critical residues in conventional MHC class I molecules that have been shown to interact with antigenic peptides. The other mouse and human NKG2D ligands are structurally similar to MIC, but lack the $\alpha 3$ domain (Fig. 2).

NKG2D ligands differ in the way they are attached to the membrane. Human ULBP1, ULBP2, ULBP3, and ULBP6 and mouse Rae-1 α – ϵ and H60c are attached to the cell surface membrane via glycosylphosphatidylinositol (GPI) anchors. Human MICA, MICB, ULBP4, and ULBP5 and mouse H60a and H60b are transmembrane proteins and have cytoplasmic tails of varying length and sequences. It has been suggested that the membrane anchorage of NKG2D ligands might impact their affinity for lipid rafts (30). Specifically, the GPI-anchored ULBP1, ULBP2, and ULBP3 glycoproteins are constitutively present in lipid rafts, whereas the transmembrane domain-containing MICA is not (30).

NKG2D ligands are highly polymorphic, particularly *MICA* and *MICB* genes for which 70 and 31 alleles have been described, respectively (<http://www.ebi.ac.uk/imgt/hla/align.html>). There is also evidence for some degree of polymorphism in the mouse *Raet1* and *H60* genes, as well as the human *RAET1* genes and promoter sequences (31,32). Interestingly, allelic variants of these ligands have been shown to bind with variable affinity to NKG2D (33,34).

Diversity of ligands driven by viral pressure

There is ample evidence of pathogens driving the diversity of NKG2D ligands. Viruses have evolved numerous mechanisms to evade NK cells (35), and in particular NKG2D-mediated viral surveillance. Most examples of NKG2D evasion mechanisms come from the study of human and mouse cytomegalovirus (HCMV and MCMV, respectively). Both HCMV and MCMV upregulate transcription of the ligands for NKG2D, which would potentially result in NKG2D-mediated lysis of infected cells by NK cells (35). As a result, viruses have deployed evasive maneuvers to prevent expression of these NKG2D ligands on the cell surface. The HCMV protein UL16 binds to ULBP1, ULBP2, ULBP6 (RAET1L), and MICB and retains these ligands intracellularly (36–42). However, UL16 is unable to bind to MICA, ULBP3, and ULBP4. Therefore, these host genes may have evolved to counter the action of UL16 and permit expression of NKG2D ligands on the surface of the HCMV-infected cell. In response, HCMV likely evolved another immunoevasin, UL142, which binds to MICA and prevents its expression via retention of full-length MICA in the cis-Golgi (43,44). Interestingly, UL142 does not bind to the *MICA**008 allele, which lacks a cytoplasmic tail, thereby making it resistant to the action of UL142. The *MICA**008 allele is frequently found in the human population, suggesting selective pressure has been exerted by HCMV on humans. Similarly to HCMV, MCMV encodes immunoevasins that prevent accessibility of mouse NKG2D ligands to the cell surface. The MCMV gene products m145, m152, and m155 selectively retain in the cytoplasm MULT1, Rae-1, and H60, respectively (45–48). In addition, m138 also downregulates H60, MULT1, and Rae-1 ϵ (49,50). These findings highlight the benefit for ligands to exhibit diversity and polymorphism in order to maintain proper recognition of infected cells by NK cells.

Expression

Normal cells

Despite the widespread agreement that NKG2D ligands are upregulated in “stressed cells”, ligand transcripts and sometimes protein can in some cases be found in normal cells. *Raet1* transcripts have been described in the embryonic brain of 129/J mice, but are absent post-birth (51). Whether Rae-1 plays a role in the embryo during development remains unknown. H60a mRNA is found in multiple tissues, including spleen, cardiac and skeletal muscle, thymus, and skin, and H60b mRNA is limited to cardiac and skeletal muscles. The most recent addition to the H60 family, H60c, is transcribed largely in the skin (26,27). Interestingly, H60a is productively expressed in BALB/c mice, but not C57BL/6 mice (hence it serves as a minor transplantation antigen), whereas H60b and H60c transcripts are detected in both C57BL/6 and BALB/c mice. MULT1 mRNA is found in the heart, thymus, lung, and kidney across most mice strains (28). The transcription of human NKG2D ligands exhibits a similar broad pattern of expression. MICA protein is expressed constitutively in intestinal epithelial cells (15). In healthy individuals, low levels of constitutive MICA expression does not result in immune cell attack of the gut; however in Crohn's and celiac autoimmune diseases, NKG2D⁺ intraepithelial lymphocytes (IELs) can attack the gut epithelium, presumably through elevated expression of MICA (52). MICA proteins and ULBP molecules are also found on both primary bronchial epithelial cells and epithelial cell lines (53). Transcripts of both MICA and MICB have recently been shown through a total body scan to be widely distributed, except in the central nervous system (54). Similarly to mouse MULT1, human ULBP transcripts appear widely expressed in humans, but lack of good staining reagents has prevented a thorough analysis of protein expression (17,18).

NKG2D ligands are also upregulated on rapidly proliferating cells. Zwirner *et al.* first reported that phytohemagglutinin (PHA) induced the expression of MICA protein in CD4⁺ and CD8⁺ T cells (55). TCR/CD3 engagement and costimulation via CD28 induced a sustained increased

expression of MICA on activated allogeneic CD4⁺ and CD8⁺ T cells (56). In addition, Cerboni *et al.* described the induction of MICA and ULBP1-3 on a fraction of dividing CD4⁺ and CD8⁺ T cells activated with the superantigen *Staphylococcus aureus* enterotoxin B (57). In mice, expression of Rae-1, and in lower amounts H60a, was detected on BALB/c, but not C57BL/6, bone marrow cells repopulating lethally irradiated recipients (58). Ovalbumin (OVA)-specific T cells activated with OVA antigen were also shown to upregulate H60 in BALB/c mice (59).

In summary, transcription of the genes encoding the human and mouse NKG2D ligands have been detected in numerous normal healthy tissues in the adult and in the normal mouse embryo. However, as discussed below post-transcriptional mechanisms exist to prevent translation and expression of these ligands in the healthy individual, presumably to avoid autoimmunity. There are conflicting reports in the literature regarding the expression of NKG2D ligand proteins in healthy, adult tissues, and some reports of protein expression in healthy tissues might be due to non-specific staining in immunohistochemistry studies. In general, there is consensus that if NKG2D ligands are expressed in normal adult tissues, it is in low amounts, possibly below the levels needed to activate immune cells expressing NKG2D receptors.

Virally infected cells

Viral infection induces the expression of NKG2D ligands, but the exact mechanism by which this occurs is for the most part unknown. Viral products could directly affect the transcriptional control of NKG2D ligands. Alternatively, infection could indirectly promote ligand expression through the induction of interferons or cytokines. As described above, the role of NKG2D has been most extensively studied following infection with mouse and human cytomegalovirus. As noted before, the MCMV and HCMV genomes encode proteins that prevent the expression of NKG2D ligands on the surface of virally infected cells. NKG2D is also involved in the control of other viral infections such as ectromelia (mousepox) virus (ECTV). In the mousepox-resistant C57BL/6 strain, depletion of NK cells results in increased viral titers and death (60). Recently, Fang *et al.* determined that NKG2D is important in the early resistance to mousepox (61). Infection of mouse embryonic fibroblasts (MEFs) *in vitro* with ECTV increased the expression of MULT1 at 18 h post-infection. In addition, *in vivo* infection with ECTV resulted in increased *Raet1* transcripts in the draining lymph nodes of infected mice, as compared with uninfected controls. Infection with a neurotropic JHM strain of mouse hepatitis virus (MHV) also lead to increased transcription of H60, MULT1, and *Raet1* in the brain of BALB/c mice (62). Furthermore, blocking NKG2D during acute MHV infection increased mortality (62). By contrast, in a mouse model of human hepatitis B virus (HBV) infection, blocking NKG2D diminished hepatitis and liver pathology (63,64). In addition, human immunodeficiency virus (HIV) infection of primary CD4⁺ T cell blasts induced the expression of ULBP1, ULBP2, and ULBP3 (65). Recently, Ward *et al.* attributed this ligand induction to the action of the HIV *vpr* gene product (66). *Vpr* was sufficient to induce ligand expression and acted through the activation of the DNA damage pathway to do so (66). Interestingly, there is evidence that the HIV protein Nef can downregulate NKG2D ligands upon infection (67). Finally, *in vitro* infection of human dendritic cells with influenza virus leads to the upregulation of ULBP1, ULBP2, and ULBP3 (68). Collectively, these findings indicate that several viruses can induce NKG2D ligands and that the NKG2D receptor on NK cells or T cells is important in the control of these infections.

Transformed cells

Many tumor cell lines and freshly extracted primary tumors express NKG2D ligands constitutively. Prior studies have established that expression of NKG2D ligands on tumors render them susceptible to killing by NK cells *in vitro* (16,69,70) and result in the *in vivo* rejection of transplantable tumors expressing these ligands (71,72). Moreover, NKG2D ligands

have been reported on a wide variety of human and mouse tumors. MICA and MICB are expressed on a subset of human hepatocellular carcinoma tissues and are involved in hepatoma cell sensitivity to NK cells (73). Tumor cells extracted from patients with different types of leukemia, including AML (acute myeloid leukemia), ALL (acute lymphatic leukemia), CML (chronic myeloid leukemia), and CLL (chronic lymphatic leukemia), express heterogeneous levels of NKG2D ligands, MICA being the most highly expressed of all (74). In addition, using the human NK cell line NKL as effector cells, Salih *et al.* showed that expression of NKG2D ligands on patient AML and CML cells rendered them susceptible to NK cell-mediated lysis in an NKG2D-dependent manner (74). Glioblastoma cells express the NKG2D ligands MICA, MICB, and ULBP1-3 mRNA (75). Interestingly, high expression of MHC class I on glioblastoma cells prevented efficient NKG2D-mediated killing of tumor cells. Killing could be restored by adenovirus-mediated over-expression of MICA in metastatic glioma cells (75). In colorectal cancer patients, high levels of MICA were associated with a good prognosis (76). Human melanoma cells express high amounts of NKG2D ligands (77), and NKG2D ligands expression was lost during the progression of uveal melanoma (78).

In mice, NKG2D ligand expression in primary tumorigenesis has not been extensively analyzed. Using the well-studied transgenic adenocarcinoma of the mouse prostate (79) (79) model of prostate cancer (79), Guerra *et al.* observed sporadic expression of NKG2D ligands on tumors (3). Interestingly, higher levels of ligands were observed on late-arising small tumors, as compared with early-arising large tumors. In addition, tumors induced in NKG2D-deficient mice expressed higher amounts of NKG2D ligands; strongly supporting the hypothesis that immunoselection by NKG2D favors the loss of NKG2D ligand expression by primary tumors. These investigators also investigated the role of NKG2D in a mouse model of lymphoid tumorigenesis, in which the E μ -myc transgene drives c-myc oncogene expression in the B cell lineage. Again, NKG2D ligands MULT1 and Rae-1 were detected at variable amounts on primary tumors, but their expression was not increased in NKG2D-deficient mice, suggesting that NKG2D is not involved in tumor progression in this setting. In summary, NKG2D ligands are expressed on the majority of tumors from essentially all cell and tissue types, and in some cases can elicit a productive immune response.

Regulation of ligands

Transcriptional regulation

The three main mechanisms by which NKG2D ligand transcription can be induced are DNA damage, TLR stimulation, and cytokine exposure. The DNA damage response pathway is involved in maintaining the integrity of the genome. The PI3K-related protein kinases ATM (ataxia telangiectasia, mutated) and ATR (ATM and Rad3 related) sense DNA lesions, specifically double-strand breaks and stalled DNA replication, respectively. This sensing results in cell-cycle arrest and DNA repair, or cell apoptosis if the DNA damage is too extensive to be repaired. This pathway has been shown to be constitutively active in human cancer cells (80–82). Gasser *et al.* provided evidence that this pathway actively regulates NKG2D ligand transcription (83). Both mouse and human cells upregulated NKG2D ligands following treatment with DNA-damaging agents. This effect was dependent on ATR function, as inhibitors of ATR and ATM kinases prevented ligand upregulation in a dose-dependent fashion. These findings provide a link between the constitutive activity of the DNA damage response in tumors (80,81) and the frequent upregulation of NKG2D ligands by these transformed cells. The exact molecular events linking the ATR/ATM-dependent recognition of DNA damage and the transcription of NKG2D ligands remain elusive.

Toll-like receptor (TLR) signaling also results in NKG2D ligand transcription. Treatment of peritoneal macrophages with TLR agonists *in vitro*, and injection of LPS *in vivo* both resulted in Rae-1 upregulation on peritoneal macrophages (84). TLR agonists increased the

transcription of *Raet1* genes but not MULT1 or H60, in a Myd88-dependent fashion. Subsequently, various groups have observed a similar effect of TLR agonists on human cells (85,86). TLR signaling on dendritic cells (DCs) also results in NKG2D ligand expression. Specifically, two groups showed the differential upregulation of NKG2D ligands, particularly ULBP1 and ULBP2, by TLR agonists such as poly(I:C) and LPS (68,87).

Cytokines can also influence NKG2D ligand expression. In particular, interferons have pleiotropic effects on NKG2D ligand expression. In humans, IFN- α leads to the expression of MICA on dendritic cells (88). By contrast, Bui *et al.* showed that IFN- α and IFN- γ treatment led to the selective downregulation of H60 on certain mouse sarcoma cells. This STAT-1-dependent effect occurred at the transcript level (89). In accordance with this study, treatment of human melanoma cells with IFN- γ resulted in decreased MICA message levels, also in a STAT-1-dependent fashion (90). Finally, transforming growth factor (TGF- β) also decreases the transcription of MICA, ULBP2, and ULBP4 on human malignant gliomas (91,92). Therefore, cytokines and interferons can differentially affect NKG2D ligand expression in different cell types and environments.

Other stimuli have also been reported to induce NKG2D ligand transcription. The *Raet1* genes were discovered because they were induced on F9 teratocarcinoma cell lines following treatment with retinoic acid (21). A retinoic acid-responsive element was mapped in the promoter of *Raet1*, suggesting a direct regulation of Rae-1 expression by retinoic acid (RA) (93). Subsequently, treatment of hepatocellular carcinoma cells with RA was also found to induce the expression of MICA and MICB (73). In addition, the promoters of *MICA* and *MICB* contain heat shock response elements, and MIC transcripts can be induced in stressed cells (94). Adenovirus E1A oncogene was also shown to upregulate NKG2D ligands on mouse and human cell lines (95). Finally, the transcription factor AP-1, which is involved in tumorigenesis and cellular stress responses, was found to regulate *Raet1* through the JunB subunit (96). Presently, transcriptional regulation of the genes encoding NKG2D ligands in humans and mice are poorly understood and this represents an important area for future investigation.

Post-transcriptional and post-translational regulation

Various mechanisms are responsible for the post-transcriptional regulation of NKG2D ligands. Stern-Ginossar *et al.* identified a group of endogenous cellular microRNAs (miRNAs) that bound to the 3'-UTR (untranslated region) of MICA and MICB (97) and repressed their translation. In addition, Yadav *et al.* identified four miRNAs that suppressed MICA expression (98). In accordance with these findings, silencing of Dicer, a key protein in the miRNA processing pathway, leads to the upregulation of MICA and MICB (99). However, in this study, upregulation of NKG2D ligands was found to be dependent on the DNA damage sensor ATM, thus suggesting that upregulation of NKG2D ligands in the absence of Dicer might be due to genotoxic stress in addition to the absence of regulatory miRNAs. In mouse cells lacking Dicer, upregulation of Rae-1 is frequently observed on splenocytes (*N. Bezman, unpublished observation*). Interestingly, HCMV was found to encode a viral miRNA, hcmv-miR-UL112, that competed with endogenous miRNA for binding to MICA and MICB 3'-UTR, thus repressing the translation of these ligands (100).

Recently, Nice *et al.* elegantly showed that MULT1 protein undergoes ubiquitination dependent on the lysines in its cytoplasmic tail, resulting in its rapid degradation (101). Ubiquitination was reduced in response to heat shock or UV irradiation, thus allowing cell surface expression of MULT1. Thus, heat shock operates on two levels: it increases the transcription of human MICA and MICB ligands, and it increases mouse MULT1 protein expression by decreasing its ubiquitination. Genotoxic stress did not affect MULT1 ubiquitination, illustrating the fact that different stimuli regulate NKG2D ligands differently.

Whether other ligands with long cytoplasmic tails are similarly regulated has not yet been investigated. The presence of multiple lysines in the cytoplasmic tail of H60a, H60b, MICA, MICB, and RAET-1G suggests that this translational control mechanism might be used by other NKG2D ligands. Interestingly, Thomas *et al.* have recently described the capacity of the KSHV (Kaposi's sarcoma-associated herpesvirus)-encoded E3 ubiquitin ligase K5 to down-regulate cell surface expression of MICA and MICB (102). In this case, ubiquitination resulted in the redistribution of MICA to the plasma membrane, rather than its targeting to degradation as observed with MULT1. Because ubiquitination is dependent on motifs in the cytoplasmic domains of the ligands, it is likely that additional posttranscriptional mechanisms are responsible for controlling the surface expression of the GPI-anchored NKG2D ligands proteins that lack cytoplasmic regions.

In addition, multiple putative regulatory motifs in the cytoplasmic domains of H60a, H60b, and MULT1 warrant further investigation. These include regulatory motifs such as the sorting/internalization motif in H60a (103). Another important mechanism of NKG2D ligand expression is through shedding from the cell surface. We will review this evasion mechanism in more detail later in this review.

Host response to membrane-bound ligands

Normal acute response to membrane ligands by immune cells

Expression of NKG2D ligands can lead to a very rapid immune response, in particular by NK cells for which NKG2D is a primary activating receptor. Ectopic expression of NKG2D ligands on tumors renders them susceptible to NK cell lysis *in vitro* (71,72). In addition, tumors bearing NKG2D ligands are rejected *in vivo*, or progress less rapidly than parental tumors (71,104). This acute rejection by NK cells is not restricted to transformed cells, as NK cells can also potentially reject Rae-1-expressing splenocytes *in vivo* ((105) and *our unpublished observation*). In addition to cell lysis, activating NK cells via NKG2D can trigger the production of cytokines, including IFN- γ , GM-CSF, and MIP-1 β (106).

NKG2D-bearing T cells also respond to cells expressing NKG2D ligands. Most studies have suggested that NKG2D plays co-stimulatory role on CD8⁺ T cells, whereas it is usually insufficient to generate a T cell response when triggered alone (107–109). However, the ability of NKG2D to costimulate T cells is dependent upon the activation state of the T cells, because in many situations engaging NKG2D on CD8⁺ T cells does not induce activation or augment TcR-induced responses (110). In accordance with these findings, we have recently investigated the response of CD8⁺ T cell isolated from mice infected with MCMV to dendritic cells expressing Rae-1 and have observed no enhancement of the T cell response (*our unpublished observation*). Therefore, why T cells can be costimulated by NKG2D in some situations, but not others, is presently unknown.

Cutaneous TCR $\gamma\delta$ ⁺ intraepithelial lymphocytes (IELs), also known as dendritic epidermal T cells (DETCs), express NKG2D (15,16). Using various mouse models of cutaneous malignancy, Girardi *et al.* showed a critical role of NKG2D⁺ DETCs for tumor recognition (111). TCR $\gamma\delta$ ⁺ T cells efficiently killed PDV tumor cells (mouse keratinocytes transformed with the carcinogen DMBA) in a NKG2D- and TCR-dependent fashion *in vitro*. Moreover, TCR δ -deficient mice exhibited increased susceptibility to PDV tumor challenge and chemically induced carcinogenesis. Recently, Whang *et al.* defined a novel NKG2D ligand named H60c, which is expressed in mouse skin (27), and observed efficient cytolysis of H60c-expressing keratinocytes by DETCs. This effect was dependent on NKG2D, as NKG2D-deficient DETCs were severely impaired in their ability to kill keratinocytes.

Strid *et al.* demonstrated rapid pleiotropic effects in the skin after Rae-1 expression (112). Using an elegantly designed “bi-transgenic” mouse in which Rae-1 can be induced in the epidermis following doxycycline treatment, they showed that in the absence of any inflammation, acute expression of Rae-1 resulted in a local immune reorganization. Within 120 h of doxycycline treatment, both Langerhans cells and DETCs exhibited changes in morphology and activation markers. The effect on DETCs morphology is unsurprising given that these cells express NKG2D and that engagement of NKG2D leads to the downstream activation of Vav-1, which has a critical role in controlling NK cell cytoskeletal polarization (113). However, Langerhans cells do not express NKG2D, and their response to Rae-1 expression is likely indirect, probably due to cytokines induced by NKG2D-mediated activation of DETCs in the tissue.

Importantly, transient NKG2D ligand expression does not always induce effector cell-mediated cytotoxicity. NKG2D-mediated crosstalk between NK cells and dendritic cells (DC) during viral infection has been suggested to augment NK cell responses (68,114,115). To investigate the effect of NKG2D ligand expression in various cell populations, we have recently generated a knock-in mouse in which a LoxP-stop-LoxP Rae-1 ϵ cassette was inserted into the *Rosa26* locus. Crossing this mouse to various tissue- or cell-restricted Cre recombinase will enable restricted Rae-1 expression into a location of interest. In particular, we are currently investigating the effects of DC-restricted expression of Rae-1 to explore the crosstalk between DC and NK cells during viral infections.

Chronic response to membrane-bound NKG2D ligands

Despite the efficient eradication of cells that express NKG2D ligands transiently, constitutive ligand expression has been shown to impair NKG2D function in humans and mice. This observation was first reported by Groh *et al.* who analyzed tumor-infiltrating lymphocytes (TILs) from human epithelial tumors (116). Presence of MICA on tumor cells consistently correlated with decreased NKG2D levels on NK cells and CD8⁺ T cells and impaired NKG2D-mediated IFN- γ production by CD8⁺ T cells. Subsequently, various groups described similarly impaired NKG2D function in patients with NKG2D ligand-expressing tumors (117–119).

Ligand-induced downregulation of the NKG2D receptor was also described by Ogasawara *et al.* in NOD mice (120). When NK cells from NOD mice were activated by IL-2, the NK cells then themselves expressed NKG2D ligands, which in turn downregulated their expression of NKG2D receptor and impaired its function. Moreover, when NK cells from C57BL/6 mice were co-cultured *in vitro* with tumor cells expressing NKG2D ligands, this again resulted in the down-modulation of NKG2D on the NK cells and impaired their NKG2D-dependent functions (120,121). Subsequently, several mouse models have been constructed to gain further understanding on the effect of sustained NKG2D engagement on receptor function (Table 1). To separate ligand expression from any other aspect of tumorigenesis or inflammation, most models developed have expressed a NKG2D ligand under a ubiquitous promoter in an otherwise normal mouse. In the majority of cases, these transgenic mice developed normally, and exhibited no sign of autoimmunity. One exception comes from a study in which a *MICB* transgene was driven by a ubiquitous promoter (122). These mice exhibited a 50% increase in the number of white blood cells, and a 10 to 20% reduction in body weight compared to their littermate control. In addition, transient exfoliation of the skin was observed at a young age. This study suggests an involvement of human MICB in skin inflammation, but it did not investigate the effect of MICB on lymphocyte populations and receptor expression, notably NKG2D on NK and CD8⁺ T cells. Moreover, off-target effects of insertion of MICB into the mouse genome might explain these unexpected results. In a separate study, Wiemann *et al.* created a mouse expressing human MICA under the mouse MHC class I H-2K^b promoter on the C57BL/6 background (123). These mice did not display overt signs of autoimmunity.

Recapitulating earlier *in vitro* observations, constitutive MICA expression resulted in the down-modulation of NKG2D on the surface of NK cells. As a result, constitutive MICA expression impaired the ability of NK cells to reject MICA-transfected RMA tumors. These investigators also investigated the effect of MICA expression on the CD8⁺ T cell response to *L. monocytogenes*. Infection of mice expressing MICA driven by the H2-K^b promoter with a *L. monocytogenes* strain recombinant for a secreted form of OVA resulted in lower percentages of IFN- γ -secreting OVA-specific CD8⁺ T cells compared to wildtype mice. Together these results showed that constitutive MICA expression results in NKG2D dysfunction on NK cells and *L. monocytogenes*-activated CD8⁺ T cells. Park *et al.* expressed human MICA under the control of the T3^b promoter, leading to restricted expression of MICA in the intestine (124). This resulted in the clonal expansion of CD4⁺, CD8 $\alpha\alpha$ -double positive IELs in the small intestine. T3^b-MICA transgenic mice developed less severe DSS-induced colitis compared to wildtype mice. These data suggest that tissue-restricted expression of MICA might lead to the development of a regulatory subset of immune cells that prevents intestinal inflammation or to the down-modulation of NKG2D, which would suppress effector T cell function. Whether NKG2D levels on lymphocytes were affected in these T3^b-MICA transgenic mice was not analyzed. In another mouse model, Oppenheim *et al.* expressed Rae-1 ϵ in FVB mice either under the involucrin promoter (inducing squamous epithelium expression) or the chicken β -actin promoter (ubiquitous expression) (105). Similarly to the findings of Wiemann *et al.*, these studies also revealed a defect in NKG2D-mediated cytotoxicity *in vivo* and an increased susceptibility to tumorigenesis. However, Rae-1 transgenic (Tg) mice generated anti-HY-specific memory T cells as effectively as wildtype controls and CD8⁺ T cells had a normal response to lymphocytic choriomeningitis virus (LCMV) at day 7 post-infection. We have generated transgenic mice in the C57BL/6 strain expressing Rae-1 ϵ under the control of the human β -actin promoter (58,110). These mice are also impaired in NKG2D-dependent functions and have increased susceptibility to Rae-1-transduced RMA tumors (*our unpublished data*). However, upon MCMV infection, Rae-1 Tg mice could efficiently generate MCMV-specific CD8⁺ T cells, despite reduced NKG2D levels on these cells (*manuscript in preparation*). Together with observations from Oppenheim *et al.*, our findings suggest that CD8⁺ T cells from Rae-1 Tg mice do not require NKG2D for the generation of effector and memory T cell functions. The defect in IFN- γ production by CD8⁺ T cells in MICA-Tg mice observed by Wiemann *et al.* in the context of *L. monocytogenes* infection might be due to impaired NKG2D function on another lymphocyte subset that influences the CD8⁺ T cell response, or simply to differential requirement for NKG2D on CD8⁺ T cells in combating viral versus bacterial infections.

Interestingly, both *in vitro* (125) and *in vivo* (105) studies have suggested that constitutive NKG2D engagement also impairs NKG2D-independent pathways, particularly missing-self recognition by NK cells. In contrast to these observations, using our Rae-1 Tg mouse models, we have observed normal NKG2D-independent responses of NK cells having been exposed to Rae-1 constitutively (*manuscript in preparation*), indicating that disrupting the NKG2D signaling pathways in NK cells and T cells does not have “off-target” effects on immune responses.

Host response to soluble ligands

Discovery of soluble NKG2D ligands in humans

In addition to membrane-bound NKG2D ligands, secreted forms of the ligands have been described in humans. In 2002, two independent groups reported secretion of MICA proteins from tumor cells and the presence of high amounts of soluble MICA (sMICA) in cancer patient sera (116,126). Secreted NKG2D ligands have been detected in a variety of cancers. Elevated amounts of soluble MICA are found in many cancers, including hematopoietic malignancies (74,126,127), epithelial cancers (116), colorectal cancer (128), liver cancer (129), prostate

cancer (130), and melanoma (131). Subsequently, MICB secretion was also described in a variety of tumors (117). Cancer cells can also shed proteins of the ULBP family. ULBP-2 is secreted both from tumor cell lines and primary tumor cells from some patients with hematopoietic malignancies (132,133). Recently, soluble ULBP2 in the sera of melanoma patients was also shown to be a strong indicator of poor prognosis (134). Although no evidence exists so far for shedding of additional ligands in human cancers, recent studies have shown that other NKG2D can be secreted from cells *in vitro*. Cerboni *et al.* developed an ELISA to measure soluble ULBP3 and found that it was secreted from mouse BaF/3 cells transfected with human ULBP3 (135). Finally, Song *et al.* also provided evidence for the existence of secreted ULBP1 protein, as detected by Western blot analysis of supernatants from gastric tumor cell lines (136).

In mice, no soluble NKG2D ligands have previously been described. We found that the mouse ligand Rae-1 could be shed from Rae-1 ϵ -transduced B16 melanoma tumor cells and from normal, healthy cells in Rae-1 ϵ Tg mice. In order to detect soluble Rae-1, we incubated cell supernatants from Rae-1 ϵ -transduced B16 melanoma cells with soluble recombinant mouse NKG2D-Ig (mNKG2D-Ig) fusion protein (or control Ig) for 30 minutes. We then used this reagent to stain human MICA-transduced BaF/3 cells. Decreased staining of MICA- BaF/3 cells indicated the presence of soluble ligand in the supernatant of the Rae-1-transduced B16 tumors. Using this assay, we found that supernatant from Rae-1 ϵ -transduced B16 melanoma cells contained a soluble protein capable of binding to mNKG2D-Ig, which is absent from the supernatant of parental B16 cells (Fig. 3A). This indicates that Rae-1 ϵ can be shed from the surface of tumor cells or secreted by the tumor cells. This effect was specific to B16 melanoma cells, because Rae-1 ϵ -transduced RMA T lymphoma cells did not exhibit the same characteristic (Fig. 3B). In addition, to determine whether Rae-1 can be secreted from cells in Rae-1 ϵ Tg mice, we compared the ability of sera from Rae-1 Tg or littermate control mice to bind to mNKG2D-Ig. We found that incubation of Rae-1 ϵ Tg sera with mNKG2D-Ig resulted in a marked decrease of mouse NKG2D-Fc binding to human MICA-transduced BaF/3 cells, as compared to serum from healthy, wildtype mice (Fig. 3C). These results suggest that Rae-1 ϵ can be shed *in vivo* in the absence of tumorigenesis. These findings are in accordance with data published on the secretion of NKG2D ligands by benign cells, such as in the context of autoimmunity (137,138), pregnancy (139,140) or SEB-activated T cells (135).

Mechanisms of generating soluble ligands

Two distinct mechanisms of generating soluble NKG2D ligands have been described. The first mechanism involves the cleavage of ligands from the cell surface by proteases. Prior studies reported that a broad-range metalloprotease inhibitor (MPI) reduced the levels of soluble MICA (sMICA) detected in tumor cell supernatants and increased the levels of surface MICA on these tumors (126). Subsequently, metalloproteases were also found to be responsible for the shedding of both soluble MICB (sMICB) and soluble ULBP2 (sULBP2) (117,133). One group reported that an inhibitor to phosphatidylinositol-specific phospholipase C (PI-PLC) increased the surface expression of GPI-anchored ULBP1 and ULBP2 on gastric tumor cell lines (136). Although these data suggest that PI-PLC might also be involved in cleaving NKG2D ligands, it is noteworthy that the investigators did not measure soluble ULBP in this assay. Thus, the increase in surface expression of NKG2D ligands might have been independent of their secretion. Recently, two groups have reported the involvement of members of the “a disintegrin and metalloproteinase” (ADAM) family in the shedding of NKG2D ligands (141,142). Many ADAM members are membrane-tethered proteases, best known for their ability to cleave ectodomains of transmembrane proteins (143). Inhibitors of ADAM10 and ADAM17 (also known as TNF α -converting enzyme, or TACE) (114) suppressed MICA and ULBP2 shedding (141). In agreement with these findings, Kohga *et al.* recently showed that chemotherapy treatment of hepatocellular carcinoma cell lines downregulated ADAM10, which led to

decreased amounts of soluble MICA in the circulation (144). In addition, ADAM17 silencing by siRNA significantly reduced shedding of MICB (142). Adding to our understanding of shedding mechanisms, Groh *et al.* showed that MICA is associated with endoplasmic reticulum protein 5 (Erp5) on the cell surface. Erp5 promotes shedding by forming transitory disulfide bonds with MICA, inducing a conformational change in the $\alpha 3$ domain of MICA (145). Interestingly, in search for metastatic-promoting factors by a forward genetic screen, Gumireddy *et al.* identified Erp5 as a protein promoting *in vivo* metastasis of breast cancer cells (146). Whether this Erp5-dependent tumor growth advantage was dependent on cleavage of NKG2D ligands from breast cancer cells was not investigated. Blocking Erp5 isomerase or ADAM protease activity might provide a therapeutic strategy to reduce secretion of NKG2D ligands by tumors.

A second mechanism to generate soluble NKG2D ligands is by alternative RNA splicing. Two groups have demonstrated the existence of alternative RNA splicing of the ULBP family of human ligands (19,147). Splice variant transcripts of ULBP5 (RAET1G) encoded by the *RAET1G2* gene, was detected in a T cell leukemia line, although in this study the presence of soluble protein in the cell supernatant was not analyzed (19). Similar to ULBP5, ULBP4 (*RAET1E*) can also be alternatively spliced to generate the soluble *RAET1E2* form (147). These studies highlight that in addition to proteolytic cleavage at the protein level, alternative splicing at the RNA level might play an important role in NKG2D immune evasion.

Mouse models to understand the consequences of soluble ligands

Until recently, most studies investigating the role of soluble NKG2D in tumorigenesis have been solely correlative. Defining the role of soluble ligands in human cancer progression is complicated by the fact that tumors secrete a variety of factors that might influence NKG2D function autonomously, such as TGF- β (148–150). The initial study suggesting an immunomodulatory role of soluble MICA (sMICA) in cancer patients showed a correlation between the presence of soluble MICA in sera of patients with MICA⁺ epithelial tumors and the level of NKG2D down-regulation on tumor-infiltrating and peripheral blood CD8⁺ T cells (116). In addition, incubation of CD8⁺ T cells with sera from patients with MICA⁺ tumors decreased the level of NKG2D on CD8⁺ T cells. However, these sera might have contained other NKG2D-modulating factors such as TGF- β . Of note however, incubation of human lymphocytes in high amounts of recombinant sMICA (100 ng/mL) did lead to a decrease in surface NKG2D expression. Using a mouse model in which human MICA was expressed under the H-2K^b promoter, Wiemann *et al.* also detected secreted MICA in the sera of the mice; however, sMICA could not downregulate NKG2D. Incubation of wildtype splenocytes with MICA-transgenic (Tg) splenocytes modulated surface NKG2D levels on wildtype splenocytes, but soluble MICA (sMICA) from MICA-Tg mice sera did not. This difference might be due to differential binding affinities of MICA to mouse and human NKG2D. In additional studies, neither sULBP2 nor sMICA/B could downregulate NKG2D levels on the human NK cell lines NKL (117,133). In this scenario, NKG2D affinity to human NKG2D ligands is not an issue. Altogether, these findings raise the important question of the physiological role of soluble NKG2D ligands during tumorigenesis.

Is tumor shedding of NKG2D ligands an efficient mechanism by which tumors evade NK cell immunosurveillance? A recent study investigated this exact question by designing a set of constructs encoding different variants of MICB. MICB was expressed either as a full-length protein (MIC), a shedding-resistant protein (MICA-A2), or a soluble protein (rsMIC). MICA-A2 contained an amino acid substitution in the $\alpha 3$ domain of MICB making it resistant to protease action. sMIC was generated by deleting the transmembrane and cytoplasmic regions of MICB. The authors transduced a prostate tumor model TRAMP-C2 (TC2) cell line with the different constructs and showed that shedding-resistant MIC-A2 prevented TC2 tumor

formation, whereas sMIC allowed for faster TC2 tumor growth. These findings support the hypothesis that soluble NKG2D ligands secreted by tumor cells can enhance tumor growth *in vivo*. Following these findings, the authors hypothesized that a feasible way of inhibiting tumor growth would be to prevent MICA shedding *in vivo*. Because blocking ERp5 or the ADAM proteases would have pleiotropic effects, the authors suggested blocking the site on MICA that is recognized by the ERp5 isomerase. In a recent paper, the authors identified a 6 amino acid motif in the $\alpha 3$ domain of MICA that is critical for its interaction with ERp5, but dispensable for MICA recognition by NKG2D (151). Future efforts should be placed in developing small molecules inhibitors or blocking antibodies to prevent MICA shedding.

To investigate whether antibody blocking of secreted ligands might restore NKG2D function, we developed a model in which MULT1 would be in the soluble form, while tumors would express a membrane-bound Rae-1 ligand. That way, blocking of the soluble MULT1 using neutralizing antibodies against MULT1 would not impair recognition of tumors expressing cell surface-bound Rae-1. We created a truncated MULT1 construct by adding a STOP codon before the transmembrane and cytoplasmic domains (Fig. 4A). The resulting construct (sMULT1) was compared with the full-length construct (FL MULT1) in all studies. We transfected 293T cells with either sMULT1 or FL MULT1 constructs. After 48 h, we harvested the supernatant and removed cell debris by centrifugation. To test for the presence of sMULT1, we incubated supernatants with mouse NKG2D-Ig fusion protein and then used this reagent to stain human MICA-transduced BaF/3 cells (mouse NKG2D binds to human MICA ligands). Soluble MULT1 in the supernatant effectively bound mNKG2D-Ig and thus prevented staining of the MICA-transduced BaF/3 cells (Fig. 4B). In addition, we found that culturing mouse splenocytes with sMULT1 down-regulated NKG2D on NK cells, as well as $\alpha\delta^+$ T cells and CD8⁺ T cells (Fig. 4C and *data not shown*). These results indicate that soluble MULT1 can effectively decrease NKG2D surface expression on lymphocytes. Reduction of NKG2D staining of NK cells and T cells cultured in the presence of sMULT1 was due to both NKG2D receptor internalization and receptor masking as shown with acid-washing experiments to remove bound sMULT1 from the cells (Fig. 4D). Acid washing of NKG2D-bearing NK cells and T cells pre-incubated with sMULT1 resulted in increased receptor expression, but not back to the level of control cells not exposed to sMULT1. We also asked whether sMULT1 could impair NKG2D-dependent cytotoxicity. We performed a standard chromium release cytotoxicity assay using as effectors IL-2 grown mouse NK cells pre-incubated with supernatant from 293T cells transfected with sMULT1 or FL MULT1. As targets, we used Rae-1-BaF/3, MICA-BaF/3, and MULT1-BaF/3 cells, which express varying amounts of NKG2D ligands, which bind to mouse NKG2D-Ig with different affinities (Fig. 5A). We found that sMULT1 decreased NK cell killing of these targets in a manner proportional to the amount of ligand present on the target cells (Fig. 5B), whereas supernatants from 293T cells transfected with a FL MULT1 construct did not affect NK cell killing due to the absence of soluble MULT1 in these cultures.

Finally, we tested the ability of anti-MULT1 monoclonal antibodies to reverse the block in NKG2D-dependent cytotoxicity mediated by sMULT1. Addition of an anti-MULT1 antibody during the cytotoxicity assay fully reversed the impaired killing of MICA-BaF/3 cells and partially reversed the impaired killing of MULT1-BaF/3 cells, presumably due to binding of the antibody to cell surface MULT1 on these MULT1-BaF/3 target cells. Interestingly, sMULT1 had no effect on killing of BaF/3 cells transduced with MCMV m157, the ligand for the activating Ly49H receptor on mouse NK cells, suggesting that NKG2D engagement in this model does not cross-tolerize other NK cell activating receptors such as Ly49H (Fig. 5C).

Concluding remarks

Despite being one of the most extensively studied activating NK receptors, NKG2D maintains many elusive aspects. Not only are new MHC-class-I-related ligands and ligand polymorphisms regularly being described, but there is now evidence for new ligand isoforms, such as RAET1E2 and RAET1G2. The list of stimuli that induce NKG2D ligand expression is also large and growing. The specific molecular players linking the actual stimuli to the transcription of these ligands is not well understood. For example, despite strong evidence that the ATM/ATR DNA damage pathway leads to transcription of human and mouse NKG2D ligands (83), the transcriptional regulators that control the promoter of NKG2D ligands are unknown. A detailed characterization of the promoter regions of NKG2D ligands will be critical to advance our understanding of the transcriptional mechanisms controlling their expression.

Probably best understood is the signaling mechanism of the NKG2D receptor. We know a lot about the molecular players that link receptor triggering to downstream effector functions, namely cytotoxicity and cytokine production. However, it has become increasingly apparent that this cytotoxic receptor is under very stringent control, and that that exposure to too much ligand or too long exposure to ligands can have detrimental effects on NKG2D-mediated signaling. This leaves us with the challenge of understanding the tipping point between immune activation and immune suppression.

Once this transition point is better defined, the manipulation of ligand expression shows many promises therapeutically. Patients that lack ligand expression altogether in their tumors or pathogen-infected cells, as a result of viral immunoevasins or tumor escape variants, will benefit from ligand-inducing treatments, such as TLR agonists, DNA-damaging agents (for example in the setting of chemotherapy in tumor patients), or treatment with TGF- β antagonists (TGF- β is a known downmodulator of both NKG2D ligands and the NKG2D receptor). On the other hand, patients with constitutively high expression of NKG2D ligands that inactivates the NKG2D receptor on NK cells and T cells, as it occurs in certain cancer patients, might benefit from drugs that reduce ligand expression or restore normal levels of NKG2D on effector cytotoxic lymphocytes. For this purpose, one could conceive the use of blocking antibodies against these NKG2D ligands. Finally, for those patients with elevated soluble NKG2D ligands in the sera, a recent growing understanding of the mechanism of ligand shedding (141,142, 144,145) and of the detrimental role of soluble ligands (Fig. 5 and (151)) show great promises for future therapies. These therapies might conceivably include the blocking of ERp5 binding to ligand (152) or blocking ERp5 isomerase function. Therefore, selectively modulating NKG2D and its ligands, and thereby the function of cytotoxic lymphocytes, may provide many opportunities to influence the outcome of infectious diseases, cancer, and certain autoimmune diseases.

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Fig 1. Schematic representation of the NKG2D receptor complex

NKG2D is a type-2 transmembrane homodimer that signals via association with adapter molecules through charged residues in the transmembrane domain. Mouse NKG2D associates with both DAP10 and DAP12 signaling molecules, whereas human NKG2D associates with DAP10 only. Pairing with DAP12 results in the phosphorylation of the immunoreceptor tyrosine-based activation motif (ITAM) and triggering of the Syk and/or Zap70 cascade. Association with DAP10 leads to tyrosine phosphorylation on the YINM motif and triggering of the PI3K and Grb2 signaling cascade.

Fig 2. Structures and affinities of mouse and human NKG2D ligands
(A) MHC class-I-chain-related protein A (MICA) and MICB are the only ligands known to date to contain an $\alpha 3$ -like domain. All other ligands contain only $\alpha 1$ and $\alpha 2$ domains. Both mouse and human NKG2D ligands can be either transmembrane-anchored proteins (shown in blue rectangle) or glycosylphosphatidylinositol (GPI)- anchored (shown in red). (B) The affinities are expressed as the equilibrium dissociation constant (K_D) and given for interactions between NKG2D receptor and ligands of the same species. Mouse NKG2D binds some human ligands (at lower affinity than mouse ligands), but human NKG2D does not bind mouse ligands.

Fig. 3. NKG2D ligands can be secreted from tumor cell lines and Rae-1 Tg mice

(A, B) Supernatant harvested from (A) B16 (black line) or Rae-1 ϵ -transduced melanoma tumor cells (red line) or (B) RMA (black line) or Rae-1 ϵ -transduced (red line) lymphoma cells was incubated with mouse NKG2D-Ig (mNKG2D-Ig) for 30 min on ice. This mixture was then used to stain MICA-transduced BaF/3 cell transfectants on ice for 30 min. A secondary goat anti-human IgG antibody was used to measure binding of mNKG2D-Ig to the transfectants. Controls included cells stained with control human Ig (grey filling) and cells stained with mNKG2D-Ig pre-incubated with soluble MULT1-secreting transfectants (dashed line). (C) Serum from Rae-1 Tg or littermate control mice was incubated with mNKG2D-Ig for 30 min on ice. This mixture was then used to stain MICA-BaF/3 cell transfectants on ice for 30 min. A secondary goat anti-human IgG antibody was used to measure binding of mNKG2D-Ig to the transfectants. Mean fluorescence intensity (MFI) of mNKG2D-Ig binding to MICA-BaF/3 cells is shown following incubation with WT or Rae-1 Tg sera.

Fig. 4. Soluble MULT1 construct is efficiently secreted and downregulates NKG2D on splenocytes (A) A soluble MULT1 (sMULT1) construct was created by inserting a STOP codon at a site between the extracellular domain and the transmembrane domain of full-length MULT1 (FL MULT1) and cloning the PCR product into pEF-bOS vector. Constructs encoding sMULT1 or FL MULT1 were transfected into 293T cells. 48 h post-transfection, supernatant from sMULT1 or FL MULT1 transfectants was collected. (B) sMULT1 (red line) or FL MULT1 (blue line) supernatant was incubated with 10 μ g/mL of mNKG2D-Ig for 30 min on ice. This mixture was then used to stain MICA-BaF/3 cell transfectants. Control human Ig staining is shown (grey filling). (C) sMULT1 (right plot) or FL MULT1 (left plot) supernatant was added to splenocytes overnight. NKG2D expression on NK cells was detected by using anti-NKG2D (CX5) antibody. (D) Mouse NKG2D-transfected BaF/3 cells were incubated with supernatant from untransfected 293T cells or 293T cells transfected with sMULT-1 or FL MULT1. After 5 h, cells were spun and acid-washed (pH=2.5) for 1.5 and 5 min. Cells were then washed and stained with anti-NKG2D (CX5) and anti-NK1.1 to detect NK cells.

Fig. 5. Soluble MULT1 impairment of NKG2D cytotoxicity is reversed by the addition of an anti-MULT1 antibody

(A–C) NK cells were isolated from spleen by removing CD4⁺ and CD8⁺ cells using antibody-coated magnetic beads, and then positively selecting for DX5⁺ cells. Cells were grown in IL-2-containing medium (4000 IU/ml IL-2) for 5 days. (A) On day 5, NK cells were harvested, incubated with FL MULT1 (solid line) or sMULT1 (dashed line) - containing supernatant for 2 h and then used in a standard ⁵¹Cr release assay against various NKG2D-ligand bearing target cells. MICA-BaF/3 (blue line), MULT1-BaF/3 (green line), and Rae1-BaF/3 (red line) cells were blocked to varying degree by the addition of sMULT1. (B) Blocking effect of sMULT1 is proportional to the amount of ligand detected on the cell surface of targets, and measured by staining with mNKG2D-Ig. (C) NK cells cultured in IL-2 for 5 days were incubated with FL MULT1 (blue line), sMULT1 alone (red line), or sMULT1 in the presence of 20 μ M anti-MULT1 antibody (green line) for 2 h. NK cells were then used in a standard ⁵¹Cr release assay against various NKG2D-ligand bearing target cells. Black line shows background killing of untransfected BaF/3 parental cells.

Table 1

Mouse models of NKG2D ligand expression

Ligand	Promoter	Expression	Strain	Observation	Ref.
MICA	H-2K ^b	All cells	C57BL/6	<ul style="list-style-type: none"> • Impaired NKG2D cytotoxicity <i>in vitro</i> and <i>in vivo</i> • Impaired CD8⁺ T cell response to <i>L. monocytogenes</i> • sMICA in serum (~50ng/ml) 	123
MICA	T3 ^b	Intestinal epithelium	C57BL/6	<ul style="list-style-type: none"> • Increased number of TCRβ CD4CD8$\alpha\alpha$ IELs in small intestine • Reduced susceptibility to DSS-induced colitis • Diminished severity of DSS-induced colitis 	124
MICB	CAG	All cells	C57BL/6	<ul style="list-style-type: none"> • Increased number of white blood cells • Reduced body weight (30–40%) • Exfoliation of skin (d7–14 post birth) 	122
Rae-1 α	Human β -actin	All cells	FVB	<ul style="list-style-type: none"> • Impaired NKG2D function in both FVB WT and Rae1 Tg mice 	<i>Our unpub.data</i>
Rae-1 ϵ	Involucrin	Epithelium	FVB	<ul style="list-style-type: none"> • Impaired NKG2D function • Normal generation of antigen-specific memory CTL (to HY antigen) 	105
Rae-1 ϵ	Ubiquitin	All cells	FVB	<ul style="list-style-type: none"> • Impaired NKG2D function • Increased susceptibility to chemical carcinogenesis and intradermal PDV tumor growth • Normal generation of antigen-specific memory CTL (to HY antigen) 	105
Rae-1 ϵ	Human β -actin	All cells	C57BL/6	<ul style="list-style-type: none"> • Impaired NKG2D-dependent tumor rejection • Normal CD8⁺ T cell response to MCMV • Normal NK response to MCMV 	<i>Our unpub.data</i>
Rae-1 ϵ	Human β -actin	All cells	BALB/c	<ul style="list-style-type: none"> • Impaired rejection of parental BALB/c bone marrow 	58
Rae-1 ϵ	Involucrin	Tet-inducible in epithelium		<ul style="list-style-type: none"> • Conformation change in DETCs and increased CD69 levels • No decrease in NKG2D expression on DETCs • Conformation change in Langerhans cells • Rapid infiltration of unconventional TCR$\alpha\beta$⁺ cells in epidermis 	112

Ligand	Promoter	Expression	Strain	Observation	Ref.
				<ul style="list-style-type: none">• Protective role of $\gamma\gamma\delta\delta 1^{-/-}$ DETCs for tumor development• Detrimental role of Langerhans cells for tumor development	

Abbreviations: MCMV, mouse cytomegalovirus; DSS, dextran sulfate sodium; CTL, cytotoxic T lymphocytes; DETC, dendritic epidermal T cells; IEL, intraepithelial lymphocytes.