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NKG2D Receptor and Its Ligands in Host Defense ^{CME}

Lewis L. Lanier

Abstract

NKG2D is an activating receptor expressed on the surface of natural killer (NK) cells, CD8⁺ T cells, and subsets of CD4⁺ T cells, invariant NKT cells (iNKT), and $\gamma\delta$ T cells. In humans, NKG2D transmits signals by its association with the DAP10 adapter subunit, and in mice alternatively spliced isoforms transmit signals either using DAP10 or DAP12 adapter subunits. Although NKG2D is encoded by a highly conserved gene (*KLRK1*) with limited polymorphism, the receptor recognizes an extensive repertoire of ligands, encoded by at least eight genes in humans (*MICA*, *MICB*, *RAET1E*, *RAET1G*, *RAET1H*, *RAET1I*, *RAET1L*, and *RAET1N*), some with extensive allelic polymorphism. Expression of the NKG2D ligands is tightly regulated at the level of transcription, translation, and post-

translation. In general, healthy adult tissues do not express NKG2D glycoproteins on the cell surface, but these ligands can be induced by hyperproliferation and transformation, as well as when cells are infected by pathogens. Thus, the NKG2D pathway serves as a mechanism for the immune system to detect and eliminate cells that have undergone "stress." Viruses and tumor cells have devised numerous strategies to evade detection by the NKG2D surveillance system, and diversification of the NKG2D ligand genes likely has been driven by selective pressures imposed by pathogens. NKG2D provides an attractive target for therapeutics in the treatment of infectious diseases, cancer, and autoimmune diseases. *Cancer Immunol Res*; 3(6); 575–82. ©2015 AACR.

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No potential conflicts of interest were disclosed.

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The members of the planning committee have no real or apparent conflicts of interest to disclose.

Learning Objectives

NKG2D is an activating receptor that is expressed on natural killer (NK) cells and subsets of T cells. The receptor binds to numerous protein ligands that are upregulated and displayed on the surface in cells undergoing stress, excessive proliferation, or transformation, allowing NK cells and T cells to kill these abnormal cells and secrete cytokines to enhance the immune response. Pathogens and tumors have evolved several mechanisms to evade this immune surveillance mechanism. Upon completion of this activity, the reader should gain an appreciation for the role of the NKG2D receptor and its ligands in host defense against infectious disease and cancer and the potential for therapeutic manipulation of this pathway.

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Introduction

The response of natural killer (NK) cells and T cells to pathogens and tumors is regulated by the integration of signals from numerous receptors expressed on their cell surface that can

initiate, enhance, or suppress their effector cell functions. Although T-cell recognition and activation are dominated by the antigen-specific T-cell antigen receptors (TCR) that are generated by somatic genetic recombination, NK cells use an extensive repertoire of germline-encoded receptors, many of which are also expressed by T cells. One of the best-characterized receptors shared by NK cells and T cells is NKG2D.

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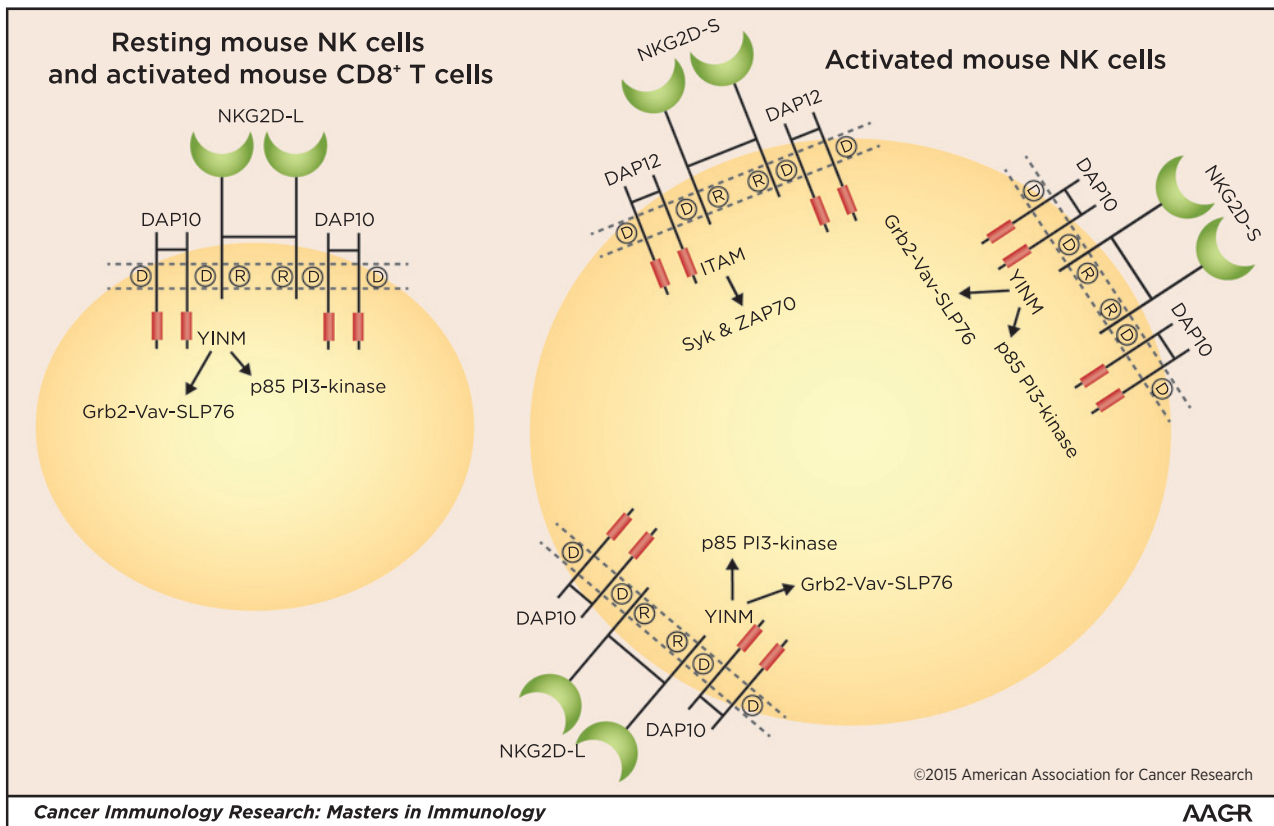
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NKG2D Genes and Proteins

An NKG2D transcript was isolated from a cDNA library prepared from a human NK-cell clone and was predicted to encode a type II transmembrane protein with a C-type

**Figure 1.**

Mouse NKG2D receptor complex. Mice express two alternatively spliced isoforms of NKG2D. NKG2D-L (long) is expressed as a disulfide-bonded homodimer that can associate with DAP10 homodimers on the surface of resting mouse NK cells and on activated (but not resting) CD8⁺ T cells. After activation, mouse NK cells express a NKG2D-S (short) isoform that can associate with homodimers of either DAP10 or DAP12. DAP10 contains a YINM motif capable of recruiting a p85 PI3-kinase, Vav-1, to the Grb2 signaling complex, whereas DAP12 contains a canonical ITAM, which can recruit Syk and ZAP70.

lectin-like extracellular domain (1). The gene encoding NKG2D, *KLRK1*, is on human chromosome 12p13.2 flanked on the centromeric side by *KLRD1* (CD94) and on the telomeric side by the cluster of *KLRC4* (NKG2F), *KLRC3* (NKG2E), *KLRC2* (NKG2C), and *KLRC1* (NKG2A) genes (2). The human *KLRK1* gene has limited polymorphism, with only two alleles that differ by a single amino acid. The mouse ortholog, *Klrk1*, is present on the syntenic region of mouse chromosome 6 and similarly has limited polymorphism (3). Orthologs of *KLRK1* are present in the genome of all mammals, as well as in marsupials, indicating that the gene is highly conserved during evolution.

Expression of NKG2D proteins on the cell surface requires its association with adapter proteins to stabilize the receptor complex (Fig. 1). Mice express two isoforms of the NKG2D protein as a result of alternative splicing. Resting mouse NK cells express a longer (NKG2D-L) protein that exclusively associates noncovalently with the DAP10 adapter protein, whereas activation of mouse NK cells induces alternative splicing of *Klrk1*, resulting in a shorter (NKG2D-S) protein isoform that can associate with either the DAP10 or DAP12 adapter protein (refs. 4, 5; Fig. 1). The association of NKG2D with DAP10 or DAP12 occurs through interactions between charged residues within the transmembrane regions of the

receptor and its adapter subunits (6). Association of NKG2D with DAP12 versus DAP10 has significant consequences for signal transduction in that DAP12 possesses a canonical immunotyrosine-based activation motif (ITAM), which recruits Syk and ZAP70 tyrosine kinases (7), whereas DAP10 has a YINM motif, which recruits a p85 PI3 kinase and Vav-1 signaling complex (6, 8). Each disulfide-bonded NKG2D homodimer associates with two DAP10 disulfide-bonded homodimers to form a hexameric receptor complex (9). Intracellular concentrations of magnesium are critical for the assembly of the NKG2D–DAP10 receptor complex in that patients with a homozygous loss of the magnesium transporter 1 (*MAGT1*) gene have impaired expression of NKG2D on the surface of their T cells and NK cells, which can be restored by dietary Mg⁺⁺ supplements (10).

Resting mouse CD8⁺ T cells do not transcribe *Klrk1*, but after TCR-induced activation, mouse CD8⁺ T cells express both NKG2D-L and NKG2D-S transcripts; however, activated mouse CD8⁺ T cell typically expresses only DAP10 and not DAP12 (refs. 4, 5, 11, 12; Fig. 1). In mice, some myeloid cell populations can transcribe (www.immgen.org) and express NKG2D on the cell surface (11, 13). In humans and mice, many $\gamma\delta$ T cells and iNKT cells and a small subset of effector or memory CD4⁺ T cells express NKG2D (11, 14). Unlike CD8⁺ T cells, TCR-mediated

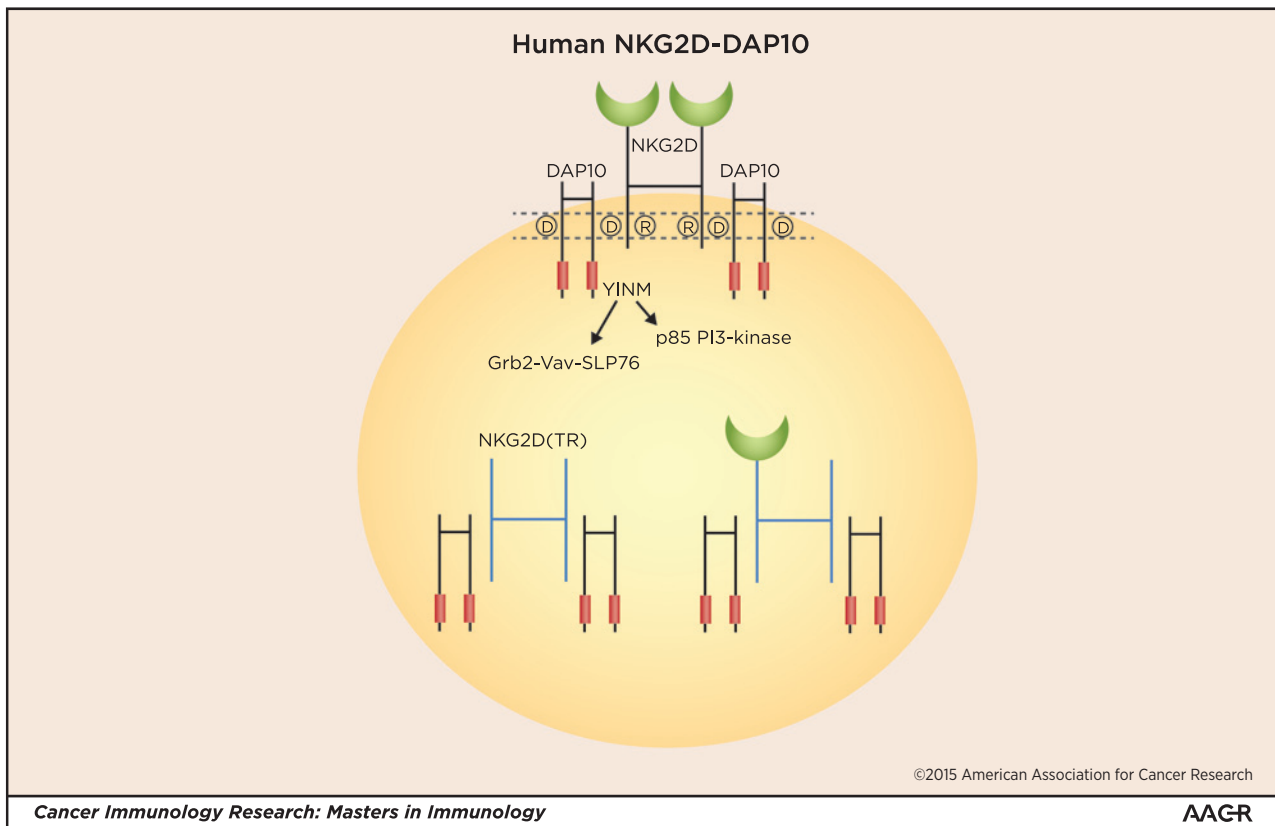


Figure 2. Human NKG2D receptor complex. Humans express a single full-length isoform of NKG2D constitutively as a disulfide-bonded homodimer on the cell surface of essentially all NK cells and CD8⁺ T cells, associated in a hexameric complex with two homodimers of the DAP10 signaling protein. Some individuals express an alternatively spliced NKG2D transcript that lacks the entire extracellular domain but retains the transmembrane and cytoplasmic domains of NKG2D (NKG2D-TR). NKG2D-TR homodimers and heterodimers of NKG2D-TR and full-length NKG2D apparently associate with DAP10 homodimers, but are retained within the cytoplasm and degraded, thereby diminishing cell surface expression of functional NKG2D complexes.

activation is not sufficient to induce NKG2D expression on CD4⁺ T cells, and the factors responsible for induction of NKG2D on CD4⁺ T cells are not known. Different than in mice, in humans, NKG2D is expressed constitutively on almost all resting CD8⁺ T cells and single-positive CD8⁺ thymocytes, as well as essentially all NK cells, and appears to associate exclusively with DAP10 in both NK cells and T cells (refs. 6, 14–16; Fig. 2). In humans, alternative splicing of *KLRK1* in NK cells and T cells can generate a truncated protein isoform [NKG2D(TR)] that lacks the extracellular domain, but this truncated protein contains the transmembrane domain and can compete with the full-length NKG2D proteins to sequester the DAP10 signaling proteins, resulting in decreased expression of functional NKG2D receptors on the cell surface (ref. 17; Fig. 2). Expression of NKG2D on NK cells and CD8⁺ T cells can be modulated by cytokines due to their effects on transcription and posttranscriptional processing of NKG2D and DAP10. In humans, IL2, IL7, IL12, and IL15 upregulate NKG2D expression (18–21), whereas TGFβ (22–24), IFNβ1 (25), and IL21 (26) downmodulate NKG2D.

NKG2D Ligand Genes and Proteins

While a single gene with limited polymorphism encodes NKG2D, this receptor recognizes a remarkably diverse array of

ligands encoded by numerous genes, some with extensive allelic polymorphism (Fig. 3). In humans, NKG2D recognizes proteins encoded by the *MICA* and *MICB* locus, which are located within the MHC on chromosome 6 near the *HLA* locus. Currently, 100 alleles of *MICA* encoding 79 protein variants and 40 alleles of *MICB* encoding 26 protein variants (<https://www.ebi.ac.uk/ipd/imgt/hla/stats.html>) have been identified in the human population. Human NKG2D also binds to another family of glycoproteins encoded by the *RAET1* (also known as *ULBP*) genes located on chromosome 6q24.2–25.3, which comprises 10 genes, *RAET1E-N*, including six loci that encode functional proteins [commonly referred to as *ULBP1* (*RAET1I*), *ULBP2* (*RAET1H*), *ULBP3* (*RAET1N*), *ULBP4* (*RAET1E*), *ULBP5* (*RAET1G*), and *ULBP6* (*RAET1L*); ref. 27]. The *RAET1* genes show less allelic polymorphism than the *MICA* and *MICB* genes. *MICA*, *MICB*, *RAET1E* (*ULBP4*), and *RAET1G* (*ULBP5*) are transmembrane-anchored glycoproteins, whereas *RAET1I* (*ULBP1*), *RAET1H* (*ULBP2*), *RAET1N* (*ULBP3*), and *RAET1L* (*ULBP6*) are glycosylphosphatidylinositol (GPI)-anchored, although *RAET1H* (*ULBP2*) may be expressed in both transmembrane-anchored and GPI-anchored forms (28), and *RAET1G* (*ULBP5*) may be GPI-anchored (29). Mice have orthologs of the human *RAET1* genes present on mouse chromosome 10, but none of the mouse ligand genes correspond to *MICA* or *MICB* or are encoded

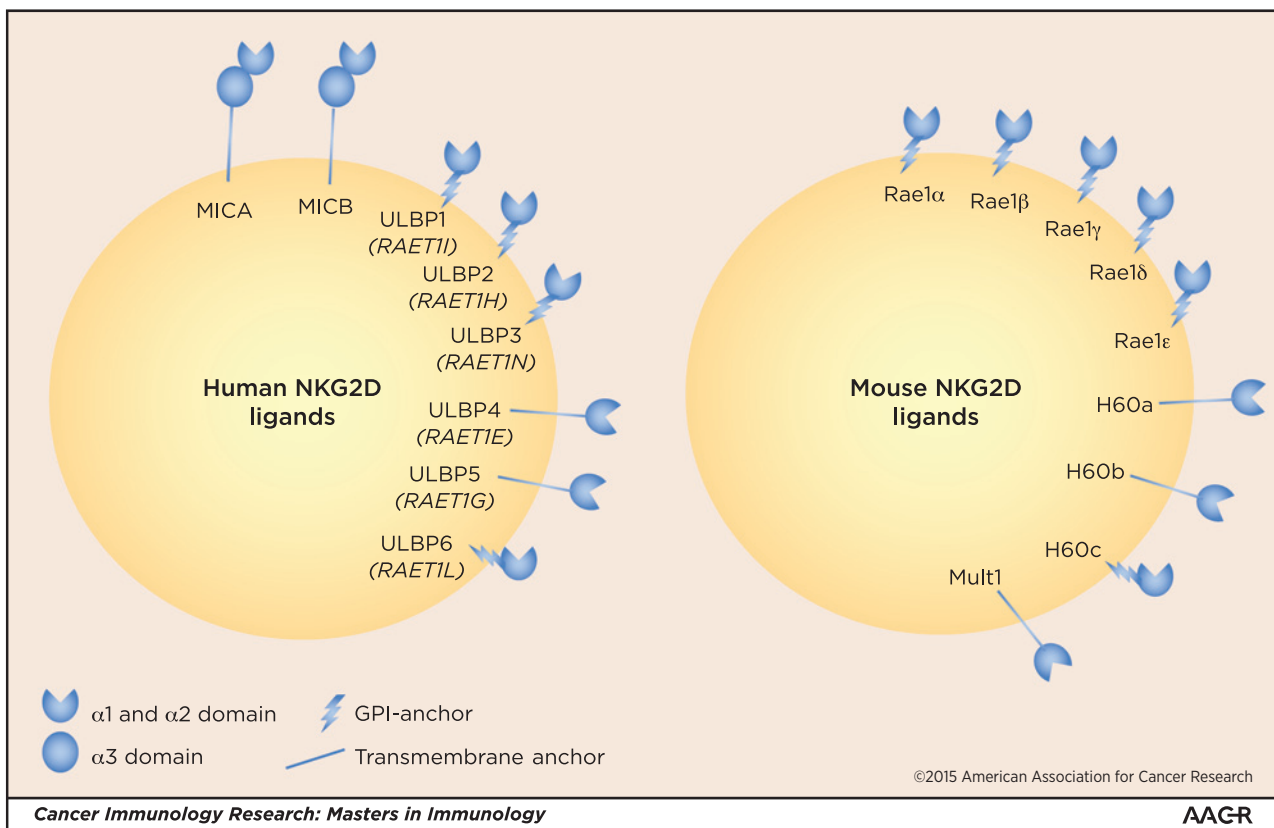


Figure 3. Schematic diagram showing all human and mouse NKG2D ligands identified to date.

within the mouse MHC. The mouse ligands include Rae1 α , Rae1 β , Rae1 γ , Rae1 δ , and Rae1 ϵ , MULT1, and H60a, H60b, and H60c, with MULT1, H60a, and H60b being transmembrane-anchored and the others GPI-anchored (30–33). All of the NKG2D ligands have α 1 and α 2 extracellular domains with homology to MHC class I molecules (MICA and MICB proteins also possess an α 3 domain), but none bind peptides or β 2-microglobulin. Structures of many of the mouse and human ligands in complex with NKG2D have been reported (reviewed in ref. 34). NKG2D binds with affinities varying from 10^{-6} to 10^{-9} mol/L to these diverse NKG2D ligands, some with only approximately 25% amino acid homology, by an "adaptive fit" mechanism, rather than inducing conformational changes in the ligands (reviewed in ref. 34).

The NKG2D ligands are regulated by transcriptional, translational, and posttranslational mechanisms (reviewed in ref. 35). Although the mouse *Rae1* genes were originally identified as being constitutively expressed in the developing embryo (36), in general, the NKG2D ligands are not expressed on the surface of healthy cells and tissues in adults. However, essentially every cell type, and every type of cancer, is capable of expressing one or more of the NKG2D ligands if appropriately stimulated. Given the existence of several distinct NKG2D ligand genes and extensive allelic polymorphisms at some of these loci, how transcription of these genes in different cell types is induced is not well defined and is likely context dependent. The induction of NKG2D ligand expression frequently is attributed to cellular

"stress," for example, in the case of infection of a cell by pathogens or cells undergoing transformation. However, normal healthy cells undergoing extensive proliferation, such as embryonic tissues, hematopoietic cells rapidly proliferating to repopulate the host after hematopoietic stem cell transplantation, and tissues undergoing wound repair, can upregulate expression of certain NKG2D ligands (36–38). Moreover, aberrant expression of NKG2D ligands has been reported in sites of inflammation and in tissues undergoing autoimmune pathologies, including rheumatoid arthritis, diabetes, celiac disease, Crohn's disease, atherosclerosis, alopecia, and asthma, although discrepant findings in various mouse model systems and human diseases have highlighted the complexity and heterogeneity of these diseases (reviewed in ref. 39). NKG2D ligand transcripts can be regulated by RNA degradation and microRNAs, and ligand proteins can be retained and degraded intracellularly by E3 ubiquitin ligases or cleaved from the cell surface by membrane matrix metalloprotease (reviewed in ref. 35). Thus, the regulation of the NKG2D ligands is a very complex process, and there are no general rules that apply for the induction of the different NKG2D ligands in different cell types.

Activation of NK Cells and T Cells by NKG2D

The prevailing concept is that NKG2D serves as a general sensor for recognition of "induced self" for the detection and elimination of hyperproliferative cells, transformed cells, or cells

infected by pathogens. As with the ligands, signaling by the NKG2D receptor in NK cells and T cells is complex and incompletely understood. Although NKG2D is expressed constitutively on essentially all resting human NK cells and CD8⁺ T cells (14), engagement of NKG2D alone is insufficient to trigger cell-mediated cytotoxicity or cytokine production (12, 40), although the simultaneous engagement of NKG2D and other "costimulatory" receptors, such as CD335 (NKp46) or CD244 (2B4), can trigger cytolytic activity in resting human NK cells (40). However, once human NK cells are "primed" by cultured in IL2 or IL15, engagement of NKG2D alone is sufficient to initiate degranulation and cytokine production. Resting mouse NK cells can be activated directly by crosslinking NKG2D *ex vivo* (11), and mice can reject normal hematopoietic cells that express a *Raet1* transgene without prior priming (37), perhaps because mouse, but not human, NKG2D associates with DAP12 in addition to DAP10. Similarly, although expressed on all naïve human CD8⁺ T cells, NKG2D fails to costimulate TCR-induced activation of resting CD8⁺ T cells (12, 19, 41), and only augments TCR-dependent activation after the T cells have been activated and cultured for a period of time *in vitro* (18, 41, 42). After some period of culture, human CD8⁺ T cells acquire the capacity to kill ligand-bearing target cells in an NKG2D-dependent, TCR-independent fashion. NKG2D-dependent, TCR-independent cytolytic activity has also been observed in intraepithelial intestinal CD8⁺ T cells isolated from patients with celiac disease, possibly due to exposure to excess IL15 in the inflamed tissue (43). How NK cells and T cells are "primed" to make NKG2D become permissive to trigger effector functions has not been defined, although cytosolic phospholipase A2 has been implicated in the process (44).

NKG2D in Immunity to Infectious Diseases and Cancer

The NKG2D system likely evolved and has been conserved to provide immunity against pathogens. NKG2D ligands are induced in cells infected with intracellular bacteria and viruses and can be induced on dendritic cells responding to microbial pathogens (reviewed in ref. 35). Again, there are no general rules for which the numerous NKG2D ligands are induced in various cell types by different pathogens. However, the importance of NKG2D is highlighted by the finding that several viruses have evolved mechanisms to prevent the expression of ligands on the cell surface of infected cells. For example, the RAET1 ligands in humans were discovered because of the ability of the UL16 glycoprotein in human cytomegalovirus (CMV) to bind to RAET1I—hence initially designated "UL16 Binding Protein 1" (ULBP1)—and to bind to MICB (45). Human CMV encodes several proteins that retain and degrade NKG2D ligands, preventing their expression on the cell surface, including UL16 targeting MICB, RAET1I (ULBP1), RAET1H (ULBP2), and RAET1L (ULBP6), US18 and US20 targeting MICA (46), UL142 targeting MICA and RAET1N (ULBP3; refs. 47, 48), and HCMV microRNA-UL112 targeting MICB transcripts (49). Mouse CMV has evolved its own viral proteins to counter NKG2D-dependent immunity. Mouse CMV m152 protein blocks surface expression of *Raet1* proteins (50, 51); m155 blocks H60 (52), m145 blocks MULT1 (53), and m138 blocks expression of MULT1 and H60 (54). The Nef protein in HIV-1 inhibits expression of MICA, RAET1I (ULBP1), and RAET1H (ULBP2; ref. 55), as well as inhib-

iting HLA-A and HLA-B. Similarly, adenovirus E3/19k (56), U21 of human herpesvirus 7 (57), and the K5 ubiquitin ligase encoded by human herpesvirus 8 (58) cause the degradation of MICA and MICB. The cowpox and monkeypox viruses secrete a soluble protein that functions as an NKG2D receptor antagonist, binding to NKG2D ligands on the surface of infected cells with high affinity to prevent detection by T cells and NK cells (59). It seems likely that the diversity of NKG2D ligand genes and their allelic polymorphism is being driven by the pathogens that are devising mechanisms to escape detection by immune cells using NKG2D.

Many cancers of all cell types express one or more of the NKG2D ligands (reviewed in ref. 60). The factors causing induction of the NKG2D ligand genes likely vary based on intrinsic features of the transformed cells, as well as influences from the microenvironment (reviewed in ref. 35). In some cases, ligands may be induced due to the genomic instability of the transformed cells, resulting in activation of the ATM and ATR DNA repair pathways (61). Induction of ligands might also be caused by hyperproliferation of the transformed cell that results in activation of E2F transcription factors (38).

Studies using transplantable mouse tumors have demonstrated that expression of an NKG2D ligand is sufficient to cause rejection of the tumor (62, 63). NKG2D plays a role in immune surveillance against primary tumors as revealed by using NKG2D-deficient TRAMP mice, a transgenic model of prostate adenocarcinoma, and NKG2D-deficient E μ -myc mice, a transgenic model of B-cell lymphoma (64). Moreover, in these tumor models, higher amounts of NKG2D ligands were present on tumors arising in NKG2D-deficient mice, suggesting NKG2D-mediated immune editing of the tumors (64).

Primary tumors frequently express NKG2D ligands, raising the question of how they avoided detection and elimination by NK cells and T cells. Several mechanisms have been identified that permit the escape of tumors bearing NKG2D ligands. These include systemic release of NKG2D ligands by tumors in cancer patients (65, 66). NKG2D ligands can be secreted from tumor cells, proteolytically cleaved from the cell surface by matrix metalloproteases, or released as exosomes (refs. 67–69; reviewed in refs. 70, 71). When NK cells or T cells encounter cells bearing NKG2D ligands, it results in downmodulation of the receptor. This is observed *in vitro* when NK cells or T cells are cocultured with NKG2D ligand-bearing cells and *in vivo* in mice bearing transgenes of NKG2D ligands. The downmodulation of NKG2D on NK cells and T cells after encounters with ligand-bearing cells likely represents a feedback mechanism to regulate the response of these lymphocytes. The ligand-induced downmodulation of NKG2D is most effectively achieved by interaction with membrane-bound, rather than soluble, NKG2D ligands, presumably because in membrane-bound form the ligands can cluster and cross-link the NKG2D receptors, triggering their internalization in the NK cells and T cells. In addition, tumor-derived factors, such as TGF β , can cause downregulation of the NKG2D receptor on NK cells and T cells (22–24). Lactose dehydrogenase 5 released from tumor cells can induce expression of NKG2D ligands on healthy monocytes, allowing them to downregulate NKG2D on NK cells and T cells and serve as decoys (72). Although it was generally thought that soluble NKG2D ligands released from tumors downregulated NKG2D on NK

cells and T cells, Deng and colleagues have reported that the high-affinity MULT1 mouse NKG2D ligand can stimulate NK cells and enhance their antitumor activity (73). Nausch and colleagues also observed that expression of NKG2D ligands on myeloid-derived suppressor cells can activate NK cells, allowing the NK cells to eliminate tumor cells lacking expression of NKG2D ligands (74). Collectively, these studies highlight the importance of the NKG2D pathway in immune surveillance of cancer.

Therapeutic Opportunities

The extent to which viruses and tumors have devised mechanisms to evade detection by NKG2D indicates that therapeutic strategies to restore or enhance NKG2D-dependent activation of NK cells or T cells may be beneficial. Alternatively, in cases where NKG2D serves to exacerbate autoimmune responses, blocking NKG2D or suppressing expression of ligands in inflammation provides an attractive therapeutic target. In the context of cancer therapy, examples include histone deacetylase inhibitors, which have been shown to induce NKG2D ligand expression on some tumor cells, and might synergize with the new generation of immune checkpoint drugs blocking CTLA-4 and PD-1 (reviewed in refs. 60, 71). Similarly, therapeutics that prevent or block the shedding of ligands by tumors or their release of NKG2D ligand-bearing exosomes might restore expression of NKG2D receptors on NK cells and T cells and improve their antitumor activity. In addition, NKG2D agonists might augment the antitumor activity of NK cells and

T cells. Chimeric antigen receptors expressing the extracellular domain of NKG2D linked to the signaling elements of CD3 ζ and DAP10 have been constructed and transduced into T cells to test their potential for cancer therapy (reviewed in ref. 75). With respect to therapeutics in infectious disease, Trsan and colleagues have introduced a cDNA encoding a NKG2D ligand into the mouse CMV genome and showed that it functions as an effective vaccine for CMV (76). These findings suggest a new strategy for the development of vaccines against pathogens that have been refractory to conventional approaches. Moreover, NKG2D agonists may boost responses against chronic pathogens, allowing their elimination. A better understanding of the cell-intrinsic and -extrinsic mechanisms that regulate the expression of the NKG2D ligands and the intracellular signals controlling NKG2D-induced responses in T cells and NK cells is needed to take full advantage of this potent immune pathway.

Disclosure of Potential Conflicts of Interest

L.L. Lanier and the University of California San Francisco have licensed intellectual property rights regarding NKG2D for commercial applications.

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