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NKG2D ligands as therapeutic targets

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The Natural Killer Group 2D (NKG2D) receptor plays an important role in protecting the host from infections and cancer. By recognizing ligands induced on infected or tumor cells, NKG2D modulates lymphocyte activation and promotes immunity to eliminate ligand-expressing cells. Because these ligands are not widely expressed on healthy adult tissue, NKG2D ligands may present a useful target for immunotherapeutic approaches in cancer. Novel therapies targeting NKG2D ligands for the treatment of cancer have shown preclinical success and are poised to enter into clinical trials. In this review, the NKG2D receptor and its ligands are discussed in the context of cancer, infection, and autoimmunity. In addition, therapies targeting NKG2D ligands in cancer are also reviewed.

Keywords: immunotherapy, MICA, Rae-1, NK cells, CD8+ T cells

Introduction

Innate immune cells recognize antigens through germline-encoded receptors and possess the ability to discriminate between self and non-self through three distinct mechanisms (1). In non-self recognition, innate leukocytes recognize conserved products of microbes not expressed by the host. In this strategy, innate leukocytes are capable of distinguishing between infectious non-self and non-infectious self. In a second strategy, recognition of "missing self" requires detection of normal self-proteins to prevent self-reactivity and attack of healthy cells. These self-proteins are specific to the host and are absent from pathogens. Recognition of altered-self constitutes a third mechanism. Innate immune cells detect markers of "abnormal self" that are upregulated due to infection or cellular transformation. The NKG2D receptor functions to recognize cells expressing induced self-proteins. Exploiting NKG2D-mediated recognition of altered self presents a method to target tumors or pathogen-infected cells.

NKG2D receptor

Expression

NKG2D is a C-type, lectin-like, type II transmembrane glycoprotein whose transcript was initially discovered in human natural killer (NK) and T cells (2, 3). Almost all NK cells in both mouse and humans express NKG2D (4). Contrary to human CD8+ T cells that constitutively express NKG2D, mouse CD8+ T cells only express the receptor after activation (4). Subsequent analyses demonstrated that the NKG2D receptor is expressed on natural killer T(NKT) cells, γδ T cells, activated mouse macrophages, and a small subset of CD4+ T cells (5). Only a small population of mouse CD4+ T cells naturally express NKG2D, and its expression on peripheral CD4+ T cells is not upregulated after T cell receptor (TCR) engagement (4). In addition, about 25% of spleen γδ T cells in the mouse express NKG2D, and it is almost ubiquitously expressed on dendritic epidermal T cells in the skin, but not by intestinal intraepithelial γδ T cells (4). Almost all peripheral blood γδ T cells in humans express NKG2D, while intestinal intraepithelial γδ T cells express low amounts of NKG2D, which is upregulated in response to IL-15 stimulation (6, 7). The expression of NKG2D is not restricted to mice and humans, and it is expressed by other mammals including rats, swine, canines, chimpanzees, and macaques.

Signaling

NKG2D, like many activating receptors, associates with an adaptor molecule to initiate signal transduction and cellular activation (Figure 1). A positively charged arginine in the transmembrane domain of NKG2D associates with a negatively charged aspartic acid in the transmembrane domain of the adaptor molecule DNAX-activating protein of 10 kDa (DAP10) (8). Although many adaptor molecules contain a cytoplasmic immunoreceptor tyrosine-based activation motif (ITAM) that mediates cell signaling, DAP10 contains a YXXM tyrosine-based motif that is similar to a motif found in the co-stimulatory molecules CD28 and ICOS (9). The YXXM motif of DAP10 recruits and activates the p85 subunit of phosphoinositide 3-kinase (PI3-K) and growth factor receptor-bound protein 2 (Grb2) (9, 10). NKG2D forms a complex with DAP10 that required to stabilize NKG2D on the cell surface (8, 9, 11). Each NKG2D homodimer associates with two homodimers of DAP10 to form a hexameric structure (8). In humans, NKG2D exclusively associates with DAP10, but two isoforms of NKG2D exist in mice as a result of alternative splicing. Activated mouse NK cells express a shorter splice-variant of NKG2D that lacks 13 amino acids in the N-terminus of the cytoplasmic domain. Unlike the longer version of NKG2D that solely binds to DAP10, NKG2D-S can bind to DAP10 or another signal transducing protein, DNAX-activating protein of 12 kDa (DAP12) (12, 13). DAP12 contains an ITAM that recruits ZAP70 and Syk to mediate NK cell activation (12, 14). NKG2D acts as a primary activation signal for NK cells and can override inhibitory signals received by other NK cell receptors (12, 13, 15). However, NKG2D receptor engagement on CD8+ T cells functions as a co-stimulatory signal by amplifying T cell activation through the TCR (15).

Regulation of NKG2D receptor expression

Many factors regulate the amount of NKG2D expressed on the cell surface. The expression and availability of DAP10 and DAP12 affect NKG2D cell surface expression because the receptor requires association with these molecules for cell surface expression. In addition, gamma-chain cytokines such as IL-2, IL-7, IL-12, and IL-15 increase cell surface expression of NKG2D in human and mouse NK and CD8+ T cells (16-18). IL-15 signaling not only regulates NKG2D expression, but increases the expression of DAP10 and phosphorylates the adaptor molecule to prime NKG2D signaling (19). Cytokines can also decrease NKG2D expression. IL-21, IFN-γ, and TGF-β have been shown

Figure 1

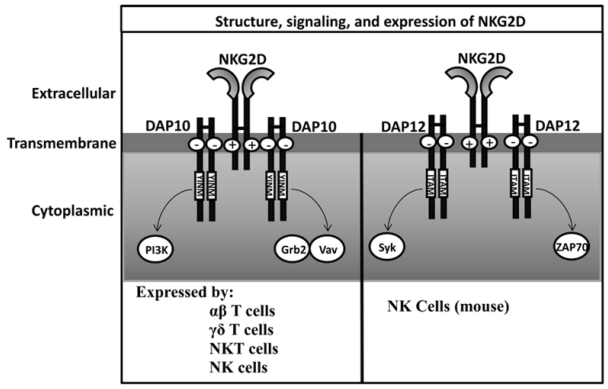

Figure 1. NKG2D receptor association with adaptor molecules and expression by leukocytes. The NKG2D receptor forms a homodimer that associates with two homodimers of the adaptor molecules DAP10 or DAP12 through interaction of a positively-charged amino acid in the transmembrane domain of NKG2D with a negatively-charged residue in the transmembrane domain of either adaptor molecule. The association of NKG2D with adaptor molecules is determined by the isoform of NKG2D expressed. Mouse and human NK cells, αβ T cells, γδ T cells, and NKT cells express a longer isoform of NKG2D that associates with DAP10. However, mouse NK cells also express a shorter splice variant of NKG2D that associate with DAP12. Upon ligand engagement, NKG2D initiate cell signaling cascades via these adaptor molecules. The DAP10-associated form of NKG2D activates PI3K and Grb2/Vav, while the DAP12-associated form activates SYK and ZAP70. NKG2D receptor engagement acts a primary activation signal for NK cells but as a co-stimulatory-type signal on T cells.

to decrease NKG2D expression (20-22). High amounts of IFN-γ decrease NKG2D expression and impair NKG2D-mediated lysis of target cells (23). IL-21 has been shown to reduce expression of DAP10 and NKG2D in human CD8+ T cells and NK cells. In mice, IL-21 stimulation of murine NK cells does not regulate NKG2D expression, and this may be due to the pairing of NKG2D with DAP12 in NK cells, which may not be regulated by IL-21 (21). Similarly, TGF-β decreases NKG2D expression, but this can be reversed after NK cells are exposed to IL-2 or IL-18 (24).

NKG2D ligands

Species specificities of NKG2D and its ligands

NKG2D ligands were discovered by treating the embryonic testicular teratoma cell line F9 with retinoic acid, which induced expression of the mouse ligand Rae-1 (25). There are currently five known members of the Rae-1 family of mouse NKG2D ligands (Rae-1α, Rae-1β, Rae-1γ, Rae-1δ, Rae-1ε). In addition, the H60 family of NKG2D ligands contains three members (H60a, H60b, H60c), while murine UL16-binding protein-like transcript 1 (MULT1) is the only member of the third family of mouse ligands. Two families of NKG2D ligands have been identified in humans. MHC class I chain-related protein A (MICA) and B (MICB) and HCMV UL16-binding proteins (ULBP1-6) are recognized by the human NKG2D receptor. All six ULBP family members are officially named *RAET1* genes and are orthologs of the mouse *Raet1* genes. Although these ligands are distant HLA class I homologues, they do not associate with β-2 microglobulin and play no known role in antigen presentation. NKG2D ligands, especially MIC genes, are highly polymorphic (26). MICA shares only 15-40% amino acid sequence identity with MHC and is encoded on the MHC locus (27). Thus, MICA alleles may be associated with specific HLA haplotypes because of linkage disequilibrium. Similar to the highly polymorphic MHC locus, there are over 60 and 20 alleles of the MICA and MICB genes, respectively (26-28). There is also evidence of polymorphism in the human *RAET1* genes and mouse *Raet1* and *H60* genes (29, 30). These allelic variants bind to NKG2D with varying affinities which may affect the threshold of NK cell triggering and T cell activation. MIC sequences have been identified in rhesus macaques that code for three genes: MIC1, MIC2, and MIC3 (31, 32). Phylogenetic analysis of these genes indicates that these are not orthologs of human MICA or MICB genes, and that they appeared to have evolved after the separation of human rhesus monkeys from a common ancestor. In addition, these alleles display polymorphism: six MIC1, five MIC2, and three MIC3 alleles have been identified (33). The RAET1 proteins are also highly polymorphic in primates. Twenty-five alleles of RAET1E/ULBP4 have been identified in the rhesus monkey (34). In chimpanzees, data suggest that MICA has been deleted and that other primates contain MICD and MICE (35). NKG2D ligands have been identified in many species and appear to serve a similar function, but there are significant differences between the ligands in protein sequence, receptor affinity, and expression patterns.

NKG2D ligand structure and binding

Human and mouse NKG2D ligands are structural homologs of MHC class I molecules. All ligands consist of two ectodomains that are distantly related to the α1 and α2 domains of the MHC class I protein. Unlike other ligands, MICA and MICB contain an α3-like domain in addition to the α1 and α2 domains (36). NKG2D displays varying affinities for its ligands (Table 1). It has the highest affinity for the murine ligand Mult1 (K_D = .004 μM), but the affinity of NKG2D for all of its ligands is about 0.02 to 1 μM, which is roughly ten-fold higher than the affinity of many immunoreceptor-ligand interactions. For example, the inhibitory receptor CTLA-4 binds to its ligand CD80 with an affinity of 0.4 μM.

Expression of NKG2D ligands by tumor cells

Many tumor cell lines and primary tumors from diverse tissue origins express NKG2D ligands. Human ULBP proteins are expressed by primary leukemia, glioma, and melanoma tumor cells (Table 2) (37-39). Almost all primary glioma isolates expressed MICA and ULBP1-3, but there was little expression of MICB on primary glioma (38). In addition, many primary tumor isolates from carcinoma (lung, breast, kidney, prostate, ovary, and colon), melanoma, and some primary leukemia cells express MICA (37-42). About 75% of primary cutaneous melanoma isolates and 50% of metastatic melanoma lesions express MICA protein (42). Moreover, human myeloma cells and over 80% of primary ovarian carcinoma cells express NKG2D ligands (43-45). NKG2D ligands on primary tumor isolates are heterogeneous with respect to the ligands found and amounts expressed, and ligand expression can also vary with tumor progression. Ovarian carcinoma cells express low amounts of the NKG2D ligands MICA, ULBP1, and ULBP3, whereas ULBP2 was more highly expressed (43). NKG2D ligands were highly expressed in lymph node metastasis of stage I colorectal cancer samples, but they were expressed in lower amounts in Stage II, III, or IV tumors (46). Higher expression of MICA in colorectal cancer patients was associated with a good prognosis (41).

Table 1
Affinity of NKG2D and other immunoreceptors.

Affinity of NKG2D and other immunoreceptors		
Immunoreceptor/Ligand	**Affinity K_D (μM)**	**Reference**
NKG2D/ NKG2D Ligands		
• *MICA/MICB*	0.5	(139, 140)
• *ULBP1*	1.1	(140)
• *Rae1ε*	0.02	(141)
• *Rae1α,β,γ,δ*	0.5	(142)
• *Mult1*	0.004	(73)
• *H60a*	0.02	(142)
• *H60b*	0.3	(72)
• *H60c*	8.7	(72)
PD-1/PD-L1	4.0	(143)
CD28/CD80	4.3	(144)
CTLA-4/CD80	0.4	(144)
TCR/pepMHC	1.0-100.0	(145)
NKp30/B7-H6	1.0	(146)
2B4/CD48	8.0	(147)

Regulation of NKG2D ligand expression

The presence of NKG2D ligands on tumor cells but not on healthy tissue implicated cellular transformation in the upregulation of ligands. It was hypothesized that oncogenes and tumor suppressor genes involved in the process of transformation may regulate NKG2D ligand expression. However, the loss of the tumor suppressor *p53* in ovarian epithelial cells, as well as forced expression of combinations of the oncogenes Kras, c-myc, or the serine/threonine kinase AKT did not regulate NKG2D ligand expression (47). The expression of NKG2D ligands can be regulated by the ATM/ATR (ataxia telangiectasia mutated/ATM- and Rad3-related) DNA damage repair pathway, but cytokine exposure and TLR stimulation may also induce NKG2D ligand transcription. Following DNA damage, the PI3-K-related proteins ATM and ATR initiate a DNA damage response. Double-stranded breaks preferentially activate ATM, while stalled DNA replication induces ATR activity. The DNA damage response has been shown to be constitutively active in advanced tumors, as well as in precancerous and early cancerous lesions, but not in healthy tissue (48, 49). Inhibiting the ATM DNA damage pathway in these murine epithelial ovarian tumor cell line T2 decreased Rae-1 expression, demonstrating the role of the DNA damage response in maintaining NKG2D ligand expression on tumor cells (47). In addition, DNA damaging agents and replication inhibitors, such as 5'Fluorouracil and cisplatin, can also induce NKG2D protein expression in an ATM/ATR-dependent manner (47). Other conventional cancer treatments, such as local ionizing radiation therapy, can upregulate expression of the mouse NKG2D ligand Rae-1 on breast carcinoma cells (Figure 2) (50). Several pharmacological drugs have been shown to induce expression of NKG2D ligands. Treatment of cell lines with the proteasome inhibitor Bortezomib (Velcade) can increase cell surface expression of NKG2D ligands. Inhibiting the proteasome has also been shown to increase ULBP2 expression, resulting in increased killing by NK cells (51). However, it is uncertain if proteasome inhibitors upregulate NKG2D ligands via DNA damage repair pathways. Inhibition of the ATM/ATR signaling pathway with caffeine did not prevent proteasome inhibitor drug-induced ULBP1 expression, indicating that inhibition of the proteasome regulates ULBP1 expression independent of the ATM/ATR DNA damage repair pathway (52). Histone deacetylase (HDAC) inhibitors have also been shown to induce DNA damage and increase expression of NKG2D ligands on tumor cells (53-55). Treatment with HDAC inhibitors resulted in increased recognition of cancer cells by cytotoxic lymphocytes via NKG2D (53, 54). Ewing's sarcoma cells treated with an HDAC inhibitor upregulated NKG2D ligands in an ATM/ATR-dependent manner, which resulted in increased sensitivity to NK cell lysis (56). It has also been demonstrated that *in vivo* treatment with all-trans retinoic acid (ATRA) or the HDAC inhibitor valproic acid in patients affected with acute myeloid leukemia (AML) led to the induction of transcription and expression of NKG2D ligands on the surface of leukemic cells (57). The tyrosine kinase receptor HER3 was also shown to regulate the expression of MICA and MICB in breast cancer cell lines (58). Similar to proteasome inhibitors, inhibition of the ATM/ATR DNA damage repair pathway with caffeine did not affect HER3 regulation of MIC expression.

Moreover, TLR signaling has been reported to induce transcription of NKG2D ligands. TLR4 and TLR7/8 agonists, but not TLR3 agonists, upregulated MIC proteins on human macrophages (59). TLR signaling through MyD88 adaptor has been shown to upregulate transcription of Rae1, but not H60 or Mult1 (59, 60). Cytokines can exert differential effects on the regulation of NKG2D ligands. Melanoma cells exposed to IFN-γ downregulate MICA and ULBP2, and IFN-γ reduces expression of mouse H60 on sarcomas (61, 62). In addition, TGF-β decreases the transcription of MICA, ULBP2, and ULBP4 on human glioma (63). The expression of NKG2D ligands has also been shown to be regulated by estradiol. Endometrial cells exposed to estradiol upregulated MICA protein (64). The MICA promoter contains an estrogen receptor response element suggesting that estrogen may increase MICA expression through transcriptional regulation.

NKG2D ligand regulation may also be mediated by post-transcriptional mechanisms. Micro-RNA encoded by human cytomegalovirus (HCMV) and overexpressed in some tumors can downregulate MICA and MICB expression by binding the 3' untranslated region (UTR) of MICA and MICB (65, 66). In the same study, it was also demonstrated that a

Table 2
Expression of NKG2D ligands on human tumor cells.

Expression of NKG2D ligands on human tumor cells	
Tumor Type	**Reference**
Carcinoma	
• *Ovarian*	(40, 43, 148-151)
• *Bladder*	(152)
• *Breast*	(40, 153-155)
• *Lung*	(40, 156, 157)
• *Hepatocellular*	(158)
• *Colon*	(40, 41)
• *Renal*	(40, 159, 160)
• *Prostate*	(40, 161)
Leukemia	
• *AML*	(39, 162-164)
• *CML*	(39, 165, 166)
• *CLL*	(167, 168)
Lymphoma	(169)
Multiple Myeloma	(138, 170)
Melanoma	(42, 171, 172)
Ewing's Sarcoma	(56, 173)
Glioma	(38, 108)
Neuroblastoma	(104)

group of endogenous micro-RNAs expressed in various human tissues and cells regulated MICA and MICB through the same mechanism (65). The effect of IFN-γ on NKG2D ligand expression may also be due to micro-RNA regulation. It has been shown that IFN-γ induces miR-520b which decreases MICA transcription by binding the 3' UTR and the MICA promoter region (67). It has also been shown that the tumor suppressive micro-RNA miR-34a/c targets the 3' UTR of ULBP2, and that the amount of mir-34 inversely correlates with the amount of ULBP2 expressed on the cell surface (68). In addition to post-transcriptional micro-RNA regulation, NKG2D ligands can be degraded post-translation. Mult1 transcripts have been shown to be highly expressed in some tissues but the protein undergoes ubiquitination and lysosomal degradation under normal conditions that prevent cell surface expression (69). These post-transcriptional regulatory mechanisms suggest that NKG2D ligands can be continually synthesized but concomitantly degraded in healthy tissue to maintain minimal cell surface ligand expression. However, regulation of the degradation process by cellular stress and transformation may be responsible for the increase in NKG2D ligand cell surface expression on infected and transformed cells. It has been shown that Mult1 cell surface expression increases after heat shock or ultraviolet stress, and that these stress responses affect ligand expression by regulating the rate of Mult1 degradation (69).

Expression of NKG2D ligands on normal cells

It is hypothesized that NKG2D ligand expression is tightly regulated in healthy adult tissue to prevent self-recognition and autoimmune reactivity. However, mRNA for these ligands is expressed in early embryos and can be detected in normal cells in some cases. NKG2D ligand transcripts were found to be expressed in the brain of embryos from 129/J mice, but transcript could not be detected post-birth (70). In humans, ULBP transcripts were also detected in the fetal brain, heart, lung, and liver (71). In healthy adult tissue isolated from mice, H60a and H60b mRNA are expressed at low levels, while the expression of H60c is restricted to the skin (72). Healthy cardiac, skeletal, spleen, liver, thymus, and skin tissue express transcript for the H60a ligand in BALB/c mice, but not in C57BL/6 mice (72). In addition, Mult1 mRNA was detected in healthy adult tissue of C57BL/6 mice (73). Similar to Mult1, ULBP transcripts in humans appear to be widely expressed in healthy adult tissue (71). ULBP transcripts were detected in the kidney, prostate, uterus, tonsil, and lymph node tissues of healthy adults (71). However, immunohistochemistry showed that most human tissues (heart, brain, liver, thyroid, lung, skin, kidney, placenta, adrenal gland, tonsil, and spleen) do not express MICA, but gastric and glandular epithelial cells do express MICA (36). Subsequent studies determined that most MIC-positive epithelial cells expressed the protein intracellularly (74). Although mRNA for NKG2D ligands is detected in healthy tissues isolated from the mouse embryo and adult tissue, data suggest that post-transcriptional regulation prevents the translation and cell surface expression of these proteins.

Expression of NKG2D ligands and self-reactivity

Recent evidence has linked the NKG2D receptor and its ligands to some autoimmune diseases. Because NKG2D receptor and ligand expression are regulated by inflammatory stimuli, sustained inflammation induced by autoreactive immune responses may contribute to NKG2D ligand expression in these tissues. Blocking the NKG2D receptor during the pre-diabetic stage in NOD mice prevented the development of diabetes (75). Because autoreactive CD8+ T cells were shown to express the NKG2D receptor and infiltrate the pancreas, it was proposed that NKG2D blockade prevented the function of autoreactive CD8+ T cells in this mouse model. NKG2D ligands have been detected on the pancreatic islets of pre-diabetic NOD mice, but age-matched BALB/c mice did not express Rae1 or other NKG2D ligands (75). Thus, the role of NKG2D in the development of type I diabetes (T1D) mellitus may be NOD strain-specific. In humans, MICA has been positively associated with human T1D through genetic linkage studies (76). It was shown that MICA polymorphisms can confer either protection or susceptibility to T1D, and that the MICA5 allele is associated with increased susceptibility to T1D when found in combination with permissive HLA class II alleles (77). In addition, MICA protein has been shown to be expressed in higher amounts in small intestine mucosa of celiac disease patients compared to healthy donors (74). In one study, intraepithelial CTLs isolated from celiac patients expressed higher amounts of NKG2D receptor, and receptor expression correlated with enhanced CTL lysis of MICA-expressing targets (78). The intraepithelial CTLs isolated from celiac patients lysed ligand-expressing targets independent of the TCR. However, Hue *et al.* found that CD8+ intraepithelial T cell lines isolated from celiac disease patients stimulated through the NKG2D receptor alone did not lyse MICA-expressing target cells in a redirected lysis assay (74). Rather, NKG2D stimulation enhanced lysis of MICA-expressing target cells only in combination with suboptimal TCR stimulation. Because intraepithelial lymphocytes (IELs) stimulated through the TCR alone mediated lysis, these data indicate that NKG2D ligands are not the primary target in celiac disease. In alopecia areata, ULBP3 was shown to be expressed on dermal sheath of the hair follicle in alopecia patients, and CD8+ NKG2D+ T cells were shown to infiltrate the hair follicle (79). However, the precise role of NKG2D in alopecia remains to be determined. In adult rheumatoid arthritis (RA), CD4+ T cells in the blood have been shown to express higher amounts of NKG2D, and proliferating synoviocytes upregulate MICA and MICB (80). In contrast, NKG2D-expressing CD4+ T cells have been shown to be immunosuppressive and their presence in juvenile onset lupus erythematosus has been shown to inversely correlate with disease activity (81). In addition, a recent study demonstrated a lack of correlation between the CD4+ NKG2D+ T cell population and RA, indicating that the NKG2D receptor may not play an integral role in RA (82).

The role of NKG2D and its ligands in autoimmune disease is complicated by linkage disequilibrium. The requirement for a certain MICA allele to be found in combination with a specific MHC allele when associated with T1D suggests that NKG2D ligands may play a secondary role in autoimmune type I diabetes, and that the disease may be MHC driven. Because MIC genes are encoded on the MHC locus, certain MHC alleles may be linked more frequently to specific MIC alleles. Therefore, when a particular MIC allele is associated with a disease, it may be the MIC gene itself or the linked MHC gene that is primarily responsible for the disease association. For example, the MICA5 allele confers susceptibility to type I diabetes, but only when inherited with permissive MHC alleles (77). Moreover, specific MHC haplotypes have been identified that are associated with autoimmune type I diabetes, rheumatoid arthritis, and celiac disease (83). The MIC alleles may play a secondary role in potentiating autoimmune inflammation after disease initiation, explaining the association with autoimmune reactivity. Blocking the NKG2D receptor after the initiation of an autoimmune response may then be a potential strategy to break the inflammatory cascade that results in healthy tissue destruction. NKG2D blockade reduced disease severity

in mildly colitic mice and attenuated colitis development when given prophylactically (84). However, NKG2D blockade during advanced stages of colitis had no effect on disease severity. These results suggest that NKG2D blockade may inhibit inflammatory cascades that promote self-reactivity, but that recognition of NKG2D ligands are not sufficient to induce disease.

NKG2D ligands in infection

NKG2D ligands can be upregulated during some bacterial and viral infections. The upregulation of ligands may be critical in alerting the immune response to infection, and may play a role in viral clearance since NK cells and CD8+ T cells express NKG2D and are often important effector cells in immunity against these pathogens. Pulmonary epithelial cells infected with *Pseudomonas aeruginosa* upregulated NKG2D ligands, and pulmonary clearance of the bacteria was dependent on NKG2D (85, 86). *Escherichia coli* and *Mycobacterium tuberculosis* rapidly increased MICA expression on the surface of intestinal epithelial cells and Rae-1; expression on macrophages, respectively (87, 88). The upregulation of MICA after *E. coli* infection was dependent on the binding of the bacterial adhesion AfaE to CD55 on epithelial cells. The rapid induction of ligands after AfaE binding is consistent with stress-induced signaling. Similarly, HIV infection induces ULBP expression on CD4+ T cell blasts (89). The HIV *vpr* gene product activated the DNA damage pathway and was sufficient to induce ligand expression. In addition, human dendritic cells(DCs) infected with influenza virus upregulated ULBP1-3 but not MICA or MICB (90). NKG2D ligands may be upregulated on antigen-presenting cells (APCs) after stimulation with pathogen-derived Toll-like receptor (TLR) ligands. LPS and poly I:C have been shown to increase NKG2D ligand expression on macrophages (60, 91-93). It has been shown that NKG2D ligands can be sequestered intracellularly during HCMV and adenovirus infections, and that HCMV impairs NK cell activation by preventing IFN-α-induced MICA and MICB upregulation on DCs (94-96). VSV, HSV, HCMV, and adenovirus infections have been shown to downregulate NKG2D ligand expression on infected cells (94-98). During HCMV infection, the UL16 glycoprotein selectively retains MICB, ULBP1, and ULBP2 in the endoplasmic reticulum and prevents cell surface expression (94, 99, 100). Infection with a UL16 deletion mutant virus resulted in NKG2D ligand upregulation on infected cells, and enhanced NK cell killing of infected targets in a NKG2D-dependent manner (100). In addition, HSV infection reduced cell surface expression of NKG2D ligands, but not the total cellular MICA content, suggesting masking, internalization, or retention of these ligands (98). Thus, targeting NKG2D ligands during some pathogen infections may promote destruction of pathogen-infected cells and clearance.

Immune evasion of NKG2D-mediated immunity

In the transgenic adenocarcinoma of the mouse prostate (TRAMP) model of spontaneous prostate adenocarcinoma, early tumors arising in mice deficient in the NKG2D receptor expressed higher amounts of NKG2D ligands compared to tumors in NKG2D-intact mice (101). However, tumor cells from mice bearing B cell lymphoma expressed heterogeneous NKG2D ligand expression in the presence or absence of NKG2D (101). These data suggest that NKG2D participates in tumor surveillance and eliminates NKG2D ligand-positive tumor cells in early tumor development in some tumor models. However, many primary tumor isolates from established tumors express MIC protein, so it is hypothesized that tumors employ mechanisms to evade NKG2D-mediated immune detection (40, 102). The release of MIC protein from the surface of tumor cells into the circulation is one mechanism hypothesized to prevent NKG2D-mediated tumor destruction (102). Soluble MIC protein has been identified in many human tumor types including breast, lung, colon, and ovarian carcinoma, glioma, neuroblastoma, leukemia, and melanoma (38, 39, 102-105). A correlation between the presence of soluble MICA in the sera of MICA-expressing tumors and the level of NKG2D downregulation on tumor infiltrating and peripheral CD8+ T cells has been shown (103). However, the sera of tumor patients contain factors that can regulate NKG2D ligand expression independent of soluble MICA. TGF-β has also been shown to decrease the transcription of MICA, ULBP2, and ULBP4 on human glioma (63). The presence of TGF-β can also downregulate NKG2D receptor expression on effector cells, and blocking TGF-β can lead to increased NKG2D expression regardless of soluble MICA present in the serum (106-108). Moreover, a mouse model created to express human MICA under the constitutive mouse H-2K^b promoter demonstrated that NKG2D receptor downregulation was not mediated by soluble MICA, but was primarily a result of persistent exposure to membrane-bound MICA (109). It is possible that soluble NKG2D ligands bind to the NKG2D receptor and prevent its interaction with membrane-bound ligands without downregulating NKG2D receptor expression. However, cytokine stimulation of cells may overcome receptor inhibition mediated by soluble ligands (24, 80). In addition, the amount of soluble MICA detected in the serum of patients with diverse cancer types is often 0.1-10 ng/ml (39, 105). The mean concentration of soluble MICA in serum from tumor-bearing patients was 228 pg/ml compared to 90 pg/ml in healthy controls (105). It was found that soluble-MIC protein downregulated NKG2D receptor expression at supraphysiological levels (100 ng/ml) (110). Although soluble MIC proteins may impair therapies targeting NKG2D ligands by immunotherapy, evidence suggests that immune effector cells are capable of eliminating NKG2D ligand-expressing tumor cells and inducing a host anti-tumor immune response (111, 112). Selective drug therapies as part of cancer treatment may also promote NKG2D-mediated tumor destruction by inducing ligand expression or reducing the cleavage of MIC proteins from the surface of tumor cells (50, 51, 53-56, 113).

The tumor microenvironment and leukocyte-mediated evasion of NKG2D-mediated anti-tumor immunity

Leukocytes within the tumor microenvironment can regulate NKG2D-mediated anti-tumor immunity. T regulatory cells (T_{regs}) can inhibit NK cell cytolytic function and IFN-γ secretion, and T_{regs} have been shown to downregulate NKG2D on human and mouse NK cells through membrane-bound TGF-β (114). In a study by Smyth *et al.*, depletion of T_{regs} resulted in the rejection of NK cell-sensitive tumors (115). In addition, NKG2D-expressing CD8+ T cells promoted elimination of B16 tumors after treatment with anti-41BB and anti-CD4 mAb to deplete regulatory T cells (116). Myeloid-derived suppressor cells (MDSCs) have also been shown to downregulate NKG2D expression and impair the cytotoxic ability of NK cells via membrane-bound TGF-β (114). However, MDSC play a dual role in anti-tumor immunity and can promote NK cell activation through NKG2D. In some tumor models, MDSC express NKG2D ligands and induce IFN-γ production in a NKG2D-dependent manner (117, 118). It has also been shown that CD4+ FOXP3+ T cells at the tumor site but not in the spleen of mice bearing ovarian carcinoma or melanoma express NKG2D ligands, and that adaptive T_{regs} in cultures of human monocytes with *M. tuberculosis* express NKG2D ligands

(118-120). NKG2D ligand expression on suppressive leukocyte populations in the tumor microenvironment indicates that NKG2D recognition may not only promote direct tumor cell lysis but may also relieve immunosuppression through lysis of regulatory leukocyte populations.

Immunotherapies targeting NKG2D ligands

Adoptive therapies transferring effector cells into tumor-bearing recipients have demonstrated therapeutic potential. NK cells, αβ T cells, γδ T cells, and NKT cells express NKG2D, and activated cells transfused into tumor-bearing recipients may induce anti-tumor immune responses and tumor destruction (121-125). Adoptive transfer of γδ T cells expressing NKG2D into patients with non-small cell lung cancer did not result in adverse events in a phase I trial (126). The *ex vivo* expansion of human PBMCs with IL-2 resulted in the outgrowth of CD8+, NKG2D+ T effector cells that lysed myeloma cells independent of their TCR (45). It was demonstrated that the cytotoxicity of these CD8+ T cells against myeloma cells was NKG2D-dependent. Because NKG2D is expressed on many transferred effector cells and can recognize its ligands on tumor cells, it is possible that NKG2D may play a part in the anti-tumor effects of adoptively transferred lymphocytes. However, direct investigation of NKG2D function was not performed in these studies. In addition, combination treatment using NKG2D-based immunotherapies with other novel immunotherapeutics or conventional treatments may improve clinical outcomes. NKG2D has been shown to contribute to anti-tumor responses elicited by IL-2 and IL-12 cytokine therapy, as well as CTLA-4 inhibitory receptor blockade (50, 127, 128). Moreover, certain drugs used in conventional treatment regimens (i.e., HDAC inhibitors, bortezomib) have been shown to upregulate NKG2D ligands on tumor cells (47, 54, 129). Therefore, irradiation, chemotherapy, or immunotherapies used in conjunction with NKG2D-based therapies may enhance tumor elimination through upregulation of NKG2D ligands on tumor cells or through activation of effector cells that eliminate the tumor through NKG2D. Because it is possible that these therapeutic agents and therapies may transiently upregulate NKG2D ligands on healthy tissue by inducing DNA damage, understanding the therapeutic window and timing of NKG2D-based therapies may be critical for safety. The expression of NKG2D ligands on many primary tumor cells makes it an attractive target for the development of novel therapeutics (Figure 2).

Figure 2

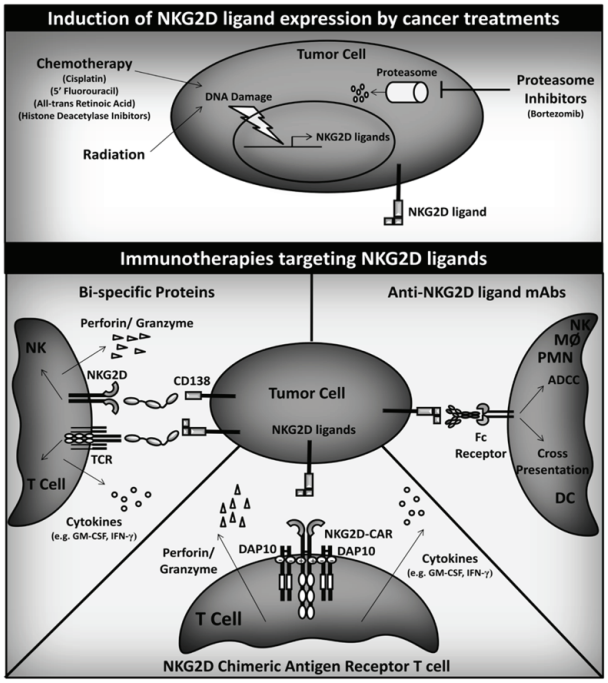

Figure 2. Induction of NKG2D ligand expression by cancer treatments and immunotherapies targeting these ligands. (top) NKG2D ligand expression increases after treatment with DNA-damage-inducing chemotherapies. In addition, local ionizing radiation or inhibition of the proteasome increases ligand expression on tumor cells. (bottom) Immunotherapies have been engineered to specifically target NKG2D ligands in cancer. NKG2D-based chimeric antigen receptor T cells express NKG2D fused to the intracellular signaling molecule CD3ζ. Upon recognition of NKG2D ligand-expressing tumor cells, NKG2D-CAR T cells lyse tumor cells through perforin/granzyme and secrete effector cytokines that modulate the tumor microenvironment. To enhance activation of T cells or NK cells, bispecific proteins targeting NKG2D ligands on tumor cells or activating NKG2D on effector cells have been developed. These bispecific proteins promote lysis of tumor cells through perforin-granzyme and secretion of effector cytokines. Targeting tumor cells with anti-NKG2D ligand monoclonal antibody therapy promotes antigen-presenting cell cross-presentation of tumor antigens to activate endogenous T cells. Moreover, anti-NKG2D ligand mAbs can promote ADCC of tumor cells by NK cells (NK), neutrophils (PMN), or macrophages (MØ) through Fc receptors. In addition to directly targeting tumor cells, therapies targeting NKG2D ligands can also eliminate regulatory leukocyte populations at the tumor site (i.e., MDSCs and Tregs).

Engineering T cells with a chimeric NKG2D receptor

The use of chimeric antigen receptor (CAR) T cells to target specific molecules on tumors has the potential to lead to long-term improved outcomes in cancer patients. Targeting NKG2D ligands with T cells engineered to express a NKG2D CAR has been shown to induce tumor elimination and long-term tumor-free survival in RMA lymphoma, ID8 ovarian carcinoma, and 5T33MM multiple myeloma (112, 130, 131). The NKG2D CAR expressing T cells consist of the full-length NKG2D receptor fused to CD3ζ and associates with DAP10. This CAR design leaves the natural NKG2D-ligand interaction and spatial dynamics between the effector and target cells intact. T cells transduced to express the NKG2D-based CAR recognize NKG2D ligands expressed on tumor cells through the CAR receptor and independent of the TCR. The NKG2D CAR signals through CD3ζ and DAP10 (132). These T cells lyse NKG2D ligand-positive tumor cells and secrete pro-inflammatory cytokines following activation (130, 133). Complete tumor elimination mediated by transfusion of NKG2D CAR T cells was not only dependent on direct killing by the transferred T cells, but also on the activation of host anti-tumor immunity (112, 130, 134). IFN-γ and GM-CSF produced by the adoptively transferred T cells transformed the tumor microenvironment from immunosuppressive to immunostimulatory and activated host lymphocytes for optimal tumor elimination (119, 130, 134, 135). In addition, treatment with NKG2D CAR T cells induced the development of a host tumor-specific T cell memory response that was capable of protecting mice from a tumor rechallenge (112, 131). Moreover, transfer of NKG2D CAR T cells has been shown to target regulatory leukocyte populations at the tumor site. CD4+ FOXP3+ T cells in the ovarian carcinoma model expressed NKG2D ligands, and transfusion of NKG2D CAR T cells reduced the number of these cells in a perforin-dependent mechanism (119). MDSC have also been shown to upregulate NKG2D ligands in some tumor models (118). Elimination of regulatory leukocyte populations

may enhance NKG2D CAR T cell-mediated anti-tumor effects. Although the presence of soluble NKG2D ligands may block the receptor, the cytolytic activity of NKG2D CAR T cells was not inhibited by physiological levels of soluble MICA protein (136). Lehner *et al.* engineered a T cell to express a CAR receptor consisting of the NKG2D ectodomain in reverse orientation fused to a CD3ζ and CD28 signaling platform. This NKG2D ligand-binding CAR enhanced T cell cytotoxicity against sarcoma tumor cell lines *in vitro* (137).

Bispecific protein therapies targeting NKG2D and NKG2D ligands

A few therapies utilizing single-chain fragment variable regions to target NKG2D or its ligands have shown therapeutic potential. A ULBP2-BB4 bispecific protein increased the susceptibility of multiple myeloma cell lines to NK cell-mediated cytotoxicity and enhanced the elimination of a xenograft tumor when combined with adoptive transfer of peripheral blood lymphocytes (138). It was shown that ULBP2 binds to the NKG2D receptor while BB4 simultaneously binds to CD138, which is overexpressed in multiple myeloma and on other tumor cells. In addition, a NKG2D/anti-CD3 scFv bispecific protein was shown to promote regression of RMA-RG lymphoma and B16F10 melanoma tumors *in vivo* (118). The NKG2D-CD3 scFv binds NKG2D ligands on tumor cells and CD3 on T cells. The host anti-tumor immune response initiated by NKG2D/anti-CD3 scFv treatment was required for tumor elimination and sustained protection from cognate tumor rechallenge. Moreover, NKG2D-CD3 scFv treatment reduced the number of local CD4+ FOXP3+ T_{regs} and Gr1+ MDSC in B16F10 melanoma-bearing mice (118).

Targeting soluble MICA

Patients treated with anti-CTLA-4 antibody blockade or vaccinated with autologous tumor cells engineered to express GM-CSF generated high-titer anti-MICA antibodies that were associated with enhanced therapeutic efficacy by relieving immunosuppression and stimulating anti-tumor cytotoxicity (128). The high-titer anti-MICA antibodies were associated with lower amounts of circulating soluble MICA and enhanced tumor cell opsonization and cross-presentation by DCs. These findings indicate that monoclonal anti-MICA antibody therapy may promote tumor elimination by neutralizing circulating MICA while simultaneously stimulating CTL activity by enhancing DC cross-presentation of tumor antigens. However, anti-MICA antibodies may also block NKG2D on effector cells from binding to NKG2D ligands on tumor cells.

Conclusion

NKG2D ligands present an attractive target for therapy. The potential expression of ligands on healthy tissue during infection and autoimmune inflammation are safety concerns. Unlike many cellular-based therapies that attempt to increase persistence of the transferred cells *in vivo*, NKG2D-targeted effector cells that persist long-term may have on-target but unwanted effects by reacting with ligands expressed on other tissues during inflammation or infection. Chronic inflammation and local cell death may invoke processes that induce NKG2D ligand expression at these sites and may contribute to pathology. However, the expression of these ligands on tumor cells from diverse origins make targeting NKG2D ligands an attractive therapy that may be broadly used in the treatment of many tumor types. The fact that cell-based NKG2D therapies recognize the target ligands independent of the MHC complex is also an advantage. The MHC-independent recognition of target cells allows the transferred effector cells to respond to tumor cells that evade immune detection through downregulation of MHC and enables these therapies to be used irrespective of MHC haplotype. Effectively harnessing the potent anti-tumor function of the NKG2D receptor while minimizing adverse effects due to ligand expression on healthy tissue will be the therapeutic hurdle for NKG2D-based cancer therapies as they enter into the clinic. Yet, the preclinical success and broad applicability of these therapies to many types of tumors offer great promise for the treatment of cancer.

Abbreviations

NK, natural killer; TCR, T cell receptor; ATM/ATR, ataxia telangiectasia mutated/ATM- and Rad3-related; DC, dendritic cell; MICA, MHC class I chain-related protein A

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