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NKG2D-Based Cancer Immunotherapy

Jennifer Wu[1] and Xuanjun Wang[2]
[1]*University of Washington*
[2]*Yunnan Agriculture University*
[1]*USA*
[2]*China*

1. Introduction

NKG2D (nature killer group 2, member D) is a C-type lectin-like activating receptor expressed by all human nature killer (NK) cells, most NKT cells, subsets of γδ T cells, and CD8 T cells. In mouse, NKG2D is also expressed by all NK cells and subsets od splenic γδ T cells and NKT cells, but only expressed by activated mouse CD8 T cells and activated mouse macrophages. NKG2D is located on a syntanic region of human choromosome 12 and on mouse chromosome 6, clustered with other NKG2 family members (Glienke et al., 1998; Ho et al., 1998) (**Figure 1**). NKG2D serves as an invariant immune activating receptor upon engagement by ligands expressed on target cells, transformed or viral infected cells. Engagement of NKG2D by its ligands can activate NK cell and co-stimulate CD8 and γδ T cells (Bauer et al., 1999; Groh et al., 2001; Wu et al., 2002). The activation signals transmitted by NKG2D can override inhibitory signals transmitted by other NK receptors. NKG2D is therefore referred as the master activating receptor for NK cells to sense cells under abnormal physiological stress. The ligands for NKG2D are not commonly present in normal tissues but can be induced under abnormal physiological condition, such as cellular transformation or viral infection. The expression pattern of NKG2D ligands in tumor cells has been extensively studied. Emerging experimental evidence have indicated that NKG2D-mediated immunity can be very effective for tumor clerance by activating NK cells, and in some cases CD8 T cells. However, it is widely accepted that NKG2D function is subverted in cancer patients, due to mechanisms of tumor immunoediting and immune suppressive effect of tumor microenvironment (**Figure 2**). Thus, inventions are in need to overcome tumor immune evasion of NKG2D immunity as an effective cancer treatment. In this chapter, we will review the basic understandings of NKG2D function in anti-tumor immunity and the challenges and advances in NKG2D-based cancer treatment.

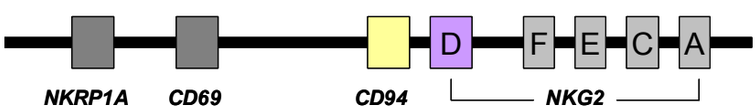

Fig. 1. The NKG2 family gene cluster. Except NKG2D, all other members of the NKG2 family form a heterodimeric complex with CD94. Different from other members, NKG2D forms a homodimer on cell surface.

2. NKG2D

2.1 Molecular structure and expression

NKG2D is a type II transmembering glycoprotein, containing C-type lectin-like domains, similar to other known NKG2 family (Eagle and Trowsdale, 2007). Although physically clustered with other NKG2 family members, NKG2D only displays 20-30% sequence homology with other members of the NKG2 family. NKG2D is highly conserved between species. For instance, human NKG2D and mouse NKG2D share 70% amino acid identity (Raulet, 2003). NKG2D was originally identified as a key activating receptor of NK cells. Subsequently NKG2D is identified on all human CD8 T cells, NKT cells, subsets of γδ T cells. In murine, NKG2D was expressed by activated and memory CD8 T cells, a proportion (25%) of splenic γδ T cells, and activated macrophages (Diefenbach et al., 2000; Mistry and O'Callaghan, 2007).

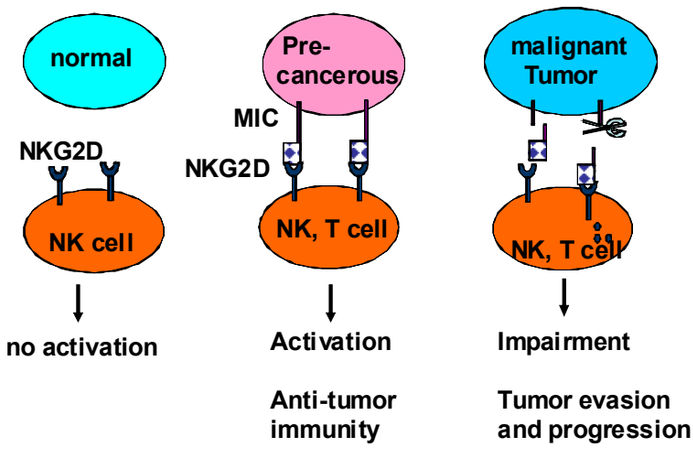

Fig. 2. Tumor cells have developed strategies to evade NKG2D immunity. The ligand of NKG2D is generally absent in normal tissues. In pre-cancerous tissues, NKG2D ligand is induced to stimulate NKG2D immunity in NK and T cells and prevents tumorigenesis. In malignant tissues, NKG2D function is impaired which allows tumor evade to immunity.

2.2 Signaling

The NKG2D molecule contains two β-sheets, two α-helices, four disulfate bonds, and a β-strand (Mistry and O'Callaghan, 2007). NKG2D forms homodimers on the cell membrane (Raulet, 2003). In both human and mouse lymphocytes, stable surface expression of NKG2D requires a complex formation of NKG2D homodimer with a Tyr-X-X-Met (YXXM) adaptor signaling molecule DAP10 at the cell membrane (Ogasawara and Lanier, 2005). Activated mouse NK cells also express a splice variant NKG2D-S, which is 13 aa shorter than normal NKG2D and signals through either DAP10 or the immunoreceptor tyrosine-based activation motif (ITAM)-containing adaptor molecule DAP12 (Long, 2002). Upon ligand engagement of NKG2D, DAP 10 is phosphorylated by src-family kinases (Figure 3), which permits the recruitment of the PI3K subunit p85 and the signaling intermediate Grb2-Vav 1 to fully activate NK cell cytotoxic pathways. In activated mouse NK cells, NKG2D-s may also independently signal through ITAM which, after phosphorylation, recruits ZAP70 (zeta-chain-associated protein kinase 70) and Syk (spleen tyrosine kinase). In NK cells, NKG2D-initiated activation signals can bypass signals transmitted through inhibitory receptors,

presumably because SHP phosphotases which are usually recruited by activation of NK inhibitory receptors do not participate NKG2D signaling (Watzl, 2003). Because of this trait, NKG2D is also regarded as the "Master" activation receptor of NK cells. Activation signal provided by NKG2D can override inhibitory signals provided by NKG2D inhibitory receptors.

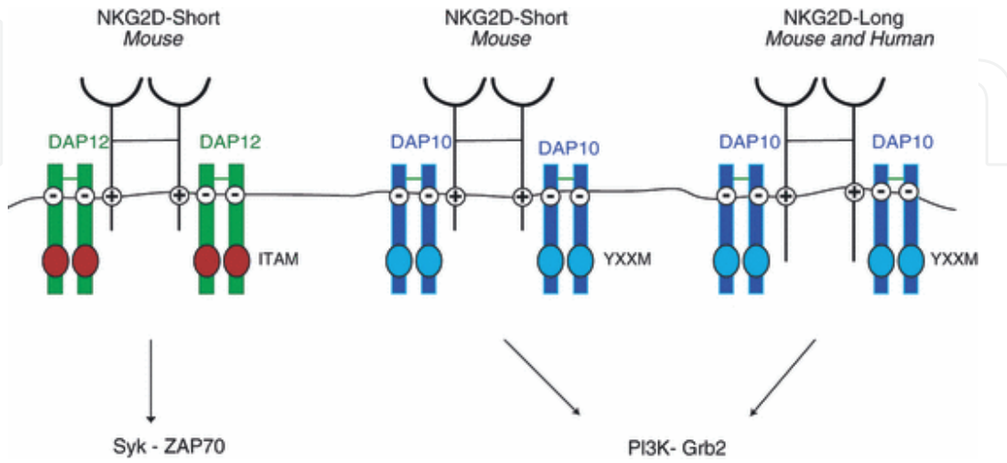

Fig. 3. NKG2D signalling pathways. Mouse NKG2D associates with both DAP10 and DAP12, whereas human NKG2D associates with DAP10 only. Adopted from Champsaur and Lanier, 2010.

3. NKG2D ligands

Multiple genes encode ligands for NKG2D have been identified in human and mice (Table 1). In human, expression of NKG2D is mostly restricted to tumor or certain viral infected cells and rarely identified in normal tissues. The expression pattern of NKG2D ligand in mouse tissues is not well understood. Nonetheless, the regulation of the NKG2D ligand expression is a delicate matter. Inappropriate expression of NKG2D ligands in normal tissues may induce autoimmune diseases, while failure to sustain surface ligand expression in transformed tissues would favor disease development and progression.

3.1 NKG2D ligands in human

Two families of NKG2D ligands are identified in humans: the MHC class I chain related family molecules A (MICA) and B (MICB) and the family of HCMV(human cytomegalovirus) UL16-binding proteins 1-6 (ULBPs 1-6) (Bahram *et al.*, 2005). All these molecules are distant HLA class I homologues but not associated with β-2 microglobulin nor have roles in antigen presentation (Eagle and Trowsdale, 2007). Althogh highly conserved within each family, members of the MIC family share little sequence or structural similarity with those of the ULBP family. The expression pattern of the MIC and ULBPs are also dissimilar.

3.1.1 Tumor-associated expression of MIC family NKG2D ligand

MIC genes are located within the MHC class I region of chromosome 6 (Bahram *et al.*, 2005). Seven MIC loci exist, but only two loci encode translated genes (MICA and MICB) (Eagle

and Trowsdale, 2007). Although MICA and MICB transcripts are widely found in normal human tissues (Schrambach et al., 2007), MICA and MICB protein are predominantly found in epithelial originated tumors, rarely expressed in normal tissue with an exception to intestinal epithelium, possibly due to the contact of these cells with intestinal microbes. MICA and MICB share over 80% amino acid identity. Both MICA and MICB are highly plymorphic. There are 51 identified MICA alleles and 23 identified MICB alleles (Bahram et al., 2005; Viny et al., 2010). To some degree, this diversity may provide protection against rapidly evolving cancers (Eagle and Trowsdale, 2007). The MIC(A/B) molecule is consisted of three extracellular domains (α1, α2, and α3), a trans-membrane region, and a cytoplasmic tail (Bahram et al., 1994; Bahram et al., 2005).

Name	Alternate Name	Cell Surface Attachment	NKG2D Affinity (K_D)
Human			
MICA	PERB11.1	Transmembrane	1 μM
MICB	PERB11.2	Transmembrane	0.8 μM
ULBP1	RAET1I	GPI anchor	1.1μM
ULBP2	RAET1H	GPI anchor or not	ND
ULBP3	RAET1N	GPI anchor	ND
ULBP4	RAET1E,LETAL	Transmembrane	ND
ULBP5	RAET1G	Transmembrane or GPI anchor	ND
ULBP6	RAET1L	GPI anchor	ND
Mice			
Rae-1α	Raet 1a	GPI anchor	690nM
Rae-1β	Raet 1b	GPI anchor	345nM
Rae-1γ	Raet 1c	GPI anchor	586nM
Rae-1δ	Raet 1d	GPI anchor	726nM
Rae-1ε	Raet e	GPI anchor	20n M
H60-a	n/a	Transmembrane	26nM
H60-b	n/a	Transmembrane	310nM
H60-c	n/a	GPI anchor	8.7μM
MULT1	n/a	Transmembrane	6 nM

Table 1. NKG2D ligands in human and mouse

3.1.2 Tumor-associated expression of ULBP family NKG2D ligand

The ULBPs were named for their ability to bind to the human cytomegalovirus UL16. protein Six members of human ULBP gene family are identified to encode functional proteins. ULBPs 1-3 and 6 are glycosylphosphatidylinositol (GPI)-linked proteins, whereas ULBPs 4 and 5 are type I transmembrane proteins (Mistry and O'Callaghan, 2007) (Figure 4). Unlike the MICs family, the ULBP family lack the α3 domain and only have the MHC class I-like α1 and α2 domains (Mistry and O'Callaghan, 2007). The expression pattern of ULBP family members are not well defined. ULBP transcripts appear widely expressed in humans (Cosman et al., 2001; Radosavljevic et al., 2002), not restrcited to transformed tissues.

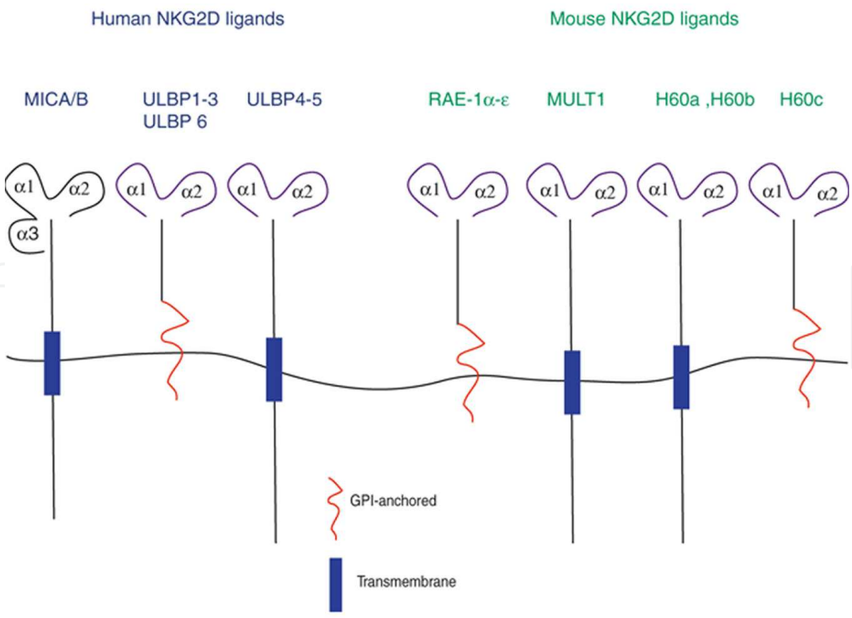

Fig. 4. Structure of NKG2D ligands in human and mice. MICA and MICB are the only known ligands containing three extracellular domains. All others (human and mouse) lack the α3 domain and are either transmembrane or GPI-anchored. Adopted from Champsaur and Lanier, 2010.

3.2 NKG2D ligands in mice

No homologue to human MIC protein was identified in mice. The identified mouse NKG2D ligands include family members of: the MHC I-related family members of retinoic acid early transcript RAE-1(α, β, γ, δ, and ε) and H60 (a, b, c), and the murine ULBP-like transcript 1 (MULT1) (Cerwenka et al., 2000; Diefenbach et al., 2003; O'Callaghan et al., 2001; Takada et al., 2008). All of these ligands only have the MHC class I-like α1 and α2 extracelluar domains. The prototype member of Rae-1 gene family was first discovered as retinoic acid (RA) early inducible cDNA clone-1 (Rae-1), which was rapidly induced on F9 teratocarcinoma cells in response to treatment with retinoic acid (Chalupny et al., 2003; Nomura et al., 1994). Presently, there are five known members of the Rae-1 family, named Rae-1α, Rae-1β, Rae-1γ, Rae-1δ, and Rae-1ε, which are differentially expressed in various mouse strains but highly related to each other (>85% identity). The H60 family comprises three members. H60a, the first ligand of the family was initially identified as a minor histocompatibility antigen by immunizing C57BL/6 mice with MHC-identical BALB.B cells (Malarkannan et al., 1998). Two novel members of H60 family were identified, and named as H60b and H60c (Takada et al., 2008). MULT1 is the unique member of the ULBP-like family of mouse NKG2D ligands and was found by database searching for mouse sequences with similarities to human ULBP (Carayannopoulos et al., 2002).

In mice, NKG2D ligand expression in primary tumorigenesis has not been extensively analyzed. Transcripts of mouse NKG2D ligand was found to be expressed in a broad range of normal tissues. H60a mRNA was found in multiple tissues, including the spleen, cardiac, skeletal muscle, thymus, and skin, whereas H60b mRNA is limited to cardiac and skeletal muscles (Zhang et al. 2010). The most recent addition to the H60 family, H60c, is transcribed

largely in the skin (Takada et al., 2008; Whang et al., 2009). H60a is productively expressed in BALB/c mice but not in C57BL/6 mice, whereas H60b and H60c transcripts are detected in both C57BL/6 and BALB/c mouse. MULT1 mRNA is found in the heart, thymus, lung, and kidney across most mice strains (Carayannopoulos et al., 2002; Takada et al., 2008). However, the expression level of NKG2D ligand on normal tissues seem to be below the threshold of inducing activate immune response to cause tissue injury.

3.3 Regulation of the NKG2D ligand expression

As NKG2D serves as the master activating receptor on NK cells, expression of NKG2D ligand NKG2D be delicatedly regulated in a pathological condition to protect normal tissue intergrity and yet maintain the alertness to diseases. The regulation is acheived at multiple levels of regulatory mechanisms, each of which will be discussed below.

3.3.1 Transcriptional regulation

The known mechanisms which regulate the NKG2D ligand transcription are mainly cellular stress, DNA damage, TLR stimulation, and cytokine exposure. The promoter region of the MICA and MICB contains contain sequences that are highly homologous to the heat shock elements of HSP70 (Venkataraman et al., 2007), a stress induced gene. Viral oncoproteins, such as adenoviral E1A protein, or cellular stress-response related products can bind to the promoter region of MICA and/or MICB to induce or upregulate its expression (Venkataraman et al., 2007). Treatment of hepatocellular carcinoma cells with RA was shown to induce the expression of MICA and MICB (Jinushi *et al.*, 2003b). The transcription factor AP-1, which is involved in tumorigenesis and cellular stress responses, was found to regulate Rae-1 transcription through the JunB subunit (Nausch *et al.*, 2006).

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 (Bartkova *et al.*, 2005; Gasser and Raulet, 2006; Gorgoulis *et al.*, 2005). Both mouse and human cells upregulate NKG2D ligands expression following treatment with DNA-damaging agents. This effect was dependent on ATR function, as inhibitors of ATR and ATM kinases can prevent ligand upregulation in a dose-dependent fashion.

TLR signaling also results in NKG2D ligand transcription in multiple mechanisms (Eissmann *et al.*, 2010). Treatment of peritoneal macrophages with TLR agonists *in vitro* and injection of LPS *in vivo* both resulted in Rae-1 upregulation on peritoneal macrophages (Hamerman *et al.*, 2004). TLR agonists increased the transcription of Raet1 genes, but not MULT1 or H60, in a Myd88-dependent fashion. TLR agonists have a similar effect on human cells (Kloss *et al.*, 2008; Nedvetzki *et al.*, 2007). TLR signaling also results in NKG2D ligand expression on DCs.

Cytokines can differentially affect NKG2D ligand expression in different cell types and environments. In humans, IFN-α leads to the expression of MICA on DCs (Jinushi *et al.*, 2003a). IFN-α and IFN-γ treatment can down-regulate H60 expression on mouse sarcoma cells(Bui *et al.*, 2006). Treatment of human melanoma cells with IFN-γ can decrease mRNA

levels of MICA in STAT-1 dependent fashion (Schwinn *et al.*, 2009). Transforming growth factor-β (TGF-β) also decreases the transcription of MICA, ULBP2, and ULPB4 on human malignant gliomas (Friese *et al.*, 2004). Macrophages cultured in the presence of IL-10 show elevated expression of MICA and MICB and ULBPs 1-3 (Schulz *et al.*, 2010).

3.3.2 Post-transcriptional regulation

Various mechanisms are responsible for the post-transcriptional regulation of NKG2D ligands. The endogenous cellular microRNAs (miRNAs) that bound to the 3'-UTR (untranslated region) of MICA,MICB and ULBP1 can repress the translation of these ligands (Stern-Ginossar *et al.*, 2008; Himmelreich *et al.*, 2011). Four miRNAs that suppressed MICA expression have been identified (Yadav *et al.*, 2009). In these findings, silencing of Dicer, a key protein in the miRNA processing pathway, leads to the upregulation of MICA and MICB (Tang *et al.*, 2008). However, miRNA-induced 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.

3.3.3 Post-translational regulation

Expression of NKG2D ligand can also be regulated post-translationally via various mechanisms. The ubiquitination on the lysines in cytoplasmic tail of MULT1 was shown to mediate its rapid degradation (Nice *et al.*, 2009). Ubiquitination can be reduced in response to heat shock or ultraviolet irradiation through the MARCH family of E3 ligases and thus allow upregulation of NKG2D ligand expression, such as MULT1 in mice and MIC (A/B) in humans (Nice *et al.*, 2010). 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. KSHV (Kaposi's sarcoma-associated herpesvirus)-encoded E3 ubiquitin ligase K5 can down-regulate cell surface expression of MICA and MICB (Thomas *et al.*, 2008). The ubiquitination may also redistribute MICA to the plasma membrane, rather than target to degradation as observed with MULT1. The sorting/internalization motif in H60a may confer the regulation mechanism (Samarakoon *et al.*, 2009). Lastly, one of the most commonly described mechanism to regulate surface NKG2D ligand expression in human cancer cells is protease-mediated shedding (Fernandez-Messina *et al.*, 2010; Liu *et al.*, 2010). This level of regulation will be discussed in details in section 6.1.

4. NKG2D in anti-tumor immunity

4.1 Evidence in experimental models

NKG2D-mediated tumor rejection has been demonstrated very effective in experimental animal models. The rejection was mediated primarily by NK cells or through a cooperation of NK cells with CD8 T cells. Overexpression of a high level of mouse NKG2D ligands Rae-1 or H60 in mouse tumor cells of various origin, including the thymoma cell line EL4, the T-cell lymphoma cell line RMA, and the poorly immunogenic and highly metastatic melanoma variant B16-BL6, induced *in vivo* rejection or retarded tumor growth when implanted into syngeneic mice (Cerwenka et al., 2001; Diefenbach et al., 2001). It was also found that the rejection of a small dose of Rae-1 or H60-expressing tumors (e.g. $1x10^4$ cells) could be achieved by NK cells or CD8 T cells alone whereas inhibition the growth of large

dose of Rae-1 or H60-expressing tumor cells (e.g. $1x10^6$ cell) required a cooperation of NK cells and CD8 T cells (Diefenbach et al., 2001).

The significance of NKG2D in controlling tumor growth was further emphasized by *in vivo* NKG2D neutralization in experimental models. When mice (B6 or balb/c background) were injected with antibody to neutralize NKG2D, these animals showed increased susceptibility to carcinogen MCA-induced fibrosarcoma in comparison to control IgG-treated mice (Smyth et al., 2005). Perhaps the most direct genetic evidence to demonstrate the role of NKG2D in tumor immunity comes from the NKG2D-deficient mice. When TRAMP mice were crossed with NKG2D-deficient mice, the progeny had 4-time increased frequency of developing poorly-differentiated tumors than $NKG2D^{WT}$ counterparts (Guerra et al., 2008).

4.2 Human cancer

Although NKG2D ligands are prevalently expressed in tumors of many types of human malignancies, there is so far no direct evidence to demonstrate the role of NKG2D in controlling tumor growth or progression. Understanding the significance of NKG2D in human cancer progression mainly comes from correlative observation in cancer patients. Massive clinical data demonstrating impaired NKG2D function in cancer patients was mediated by various mechanisms. A number of studies elegantly demonstrating the positive correlation of impaired NKG2D function with cancer disease stages. We are one of the first groups demonstrating that impaired NKG2D-mediated NK cell function correlated with cancer stages in prostate cancer patients (Wu et al., 2004). In this study, circulating NK cells were isolated from prostate cancer patients with various stages of diseases. NKG2D expression and NK cell function were analyzed *in vitro*. The result showed a gradually loss of $NKG2D^+$ NK population from patients with low grade to high grade of cancer, with complete loss of NKG2D expression on NK cells from patients with advanced diseases. As an obvious consequence, NKG2D-mediated cytotoxicity of these NK cells against tumor cells was severely subverted. Similar observations were demonstrated in the progression of other types of cancers, such as multiple myeloma and colon cancer (Doubrovina et al., 2003; Jinushi et al., 2008). In gliomas patients, tumor burden was found to be associated with deficiency of NKG2D expression on NK and CD8 T cells (Crane et al. 2010). A number of studies have also described that dysfunction of NKG2D on $CD3^+CD56^+$ NK-like T cells and subsets of γδ T cells was associated with poor prognosis of certain cancers (Bilgi et al., 2008; Marten et al., 2006; Wang et al., 2008).

5. Tumor immune evasion of NKG2D immunity

5.1 Tumor shedding of NKG2D ligand as the immune evasion mechanism

Expression of NKG2D ligand on tumors should effectively trigger immune response, at least NK cell innate response at the early stage of tumorigenesis, to eradicate tumors in human. However, in many types of established tumors of human malignancy, the NKG2D ligand MIC was highly expressed (Groh et al., 1999). The very paradoxical question is: how can human epithelial tumors develop and persist while the surface MIC molecule should identify them as abnormal and flag them for immune destruction? Clinical studies demonstrated that most of the human malignancies have developed mechanisms to evade NKG2D-mediated anti-tumor immunity. One of the common mechanisms by which human cancers evade NKG2D immunity is shedding of the NKG2D ligand MIC from tumor cell

surface to release a stable soluble form of MIC (sMIC) to the circulation (Groh et al., 2002). This mechanism has been identified in an array of human malignancies, including carcinomas of prostate, breast, lung, colon, Kidney, and ovarian, gliomas, neuroblasttomas, and melanoma (Groh et al., 2002). Elevated serum levels of sMIC has been shown to be correlative with advanced cancer stages (Doubrovina et al., 2003; Holdenrieder et al., 2006a, b; Jinushi et al., 2008; Rebmann et al., 2007; Tamaki et al., 2010; Tamaki et al., 2009; Tamaki et al., 2008; Wu et al., 2004). Some studies have suggested that serum levels of sMIC may be used as a valid prognosis factor for cancer progression (Tamaki et al.,2010 ; Tamaki et al., 2009). Tumor-derived sMIC can impose several negative imprints on host immune system. First, shedding can reduce the density of membrane-bound NKG2D lgand, namely MIC on tumor cells and thus reduce the visibility of tumor cells by the immune surveillance. Second, sMIC in the circulation can not only mask NKG2D on effector NK, NKT and T cells, but also induce NKG2D internalization (Champsaur and Lanier, 2010). Third, sMIC may induce the expansion of immune suppressive $NKG2D^{+}CD4$ T cells in the tumor microenvironment (Groh et al., 2003).

5.2 The alternative hypothesis

The hypothesis that tumor-derived sMIC is immune suppressive in cancer patients is widely accepted. Currently, an alternative hypothesis that chronic exposure to membrane-bound ligands also impairs NKG2D function was also proposed, based on several *in vitro* and *in vivo* studies. This alternative hypothesis raised a concern on the effectiveness and strategy on NKG2D-based immune therapy. The *in vitro* study was conducted by co-culturing purified mouse splenic NK cells with RAE-1-overexpressing tumor cells. The investigator found that NKG2D expression was down-regulated (Coudert et al., 2005). It was not clear in this study whether the down-regulation of prolonged in vitro culture is due to soluble RAE-1 or membrane-bound RAE-1, as RAE-1 was recently shown to be shed by mouse tumor cells (Champsaur and Lanier, 2010). With a different aspect of limitations, the existing evidence from *in vivo* studies was based on enforced ectopic constitutive expression of NKG2D ligand on normal mouse, not in the context of tissue-specific expression without resembling the feature of NKG2D ligand expression in cancer patients. For example, one transgenic mouse model that was created by expressing human MICA under the constitutive and ubiquitous mouse MHC class I $H\text{-}2K^{b}$ promoter on a C57BL/6 background showed impaired ability of NK cells to reject MICA-transfected RMA tumors in comparison to the wild-type counterparts (Wiemann et al., 2005). In other models, NKG2D ligand RAE-1ε was expressed in normal mice under the constitutive involucrin promoter (inducing squamous epithelium expression) or the ubiquitous chicken β-actin promoter; local and systemic NKG2D downregulation was noted in these mice in comparison to the wild-type counterparts (Oppenheim et al., 2005). Notably, in these transgenic mouse models, NKG2D ligand expression was "ectopic" under the direction of a constitutive or ubiquitous promoter in somatic cells. Given the magnitude of ligand-induced NKG2D signaling on activating NK cell cytoxicity, down-regulation of NKG2D function may be expected in these transgenic mice in compare to an otherwise wild type counterpart. This would be a self-regulatory mechanism in response to "a suicide machinery" to allow normal embryonic development. Thus, whether the sustained systemic ligand-induced downregulation of NKG2D in these mouse models truly represents the real situation in cancer patients should be carefully evaluated.

5.3 Does chronic exposure to membrane-bound ligand impair NKG2D function?

The alternative hypothesis raised a fatal therapeutic concern whether sustaining NKG2D ligand on tumor cell surface would be beneficial or detrimental for host anti-tumor immunity. To resolve the controversial, we constructed a mutant shedding resistant membrane-restricted NKG2D ligand MICB.A2. We overexpressed the native shedding-sensitive MICB and the mutant MICB.A2 both of which can be recognized by mouse NKG2D (Wu et al., 2009) respectively in a highly tumorigenic mouse prostate tumor cell line TRAMP-C2 and implanted these cell lines into SCID mice. Interestingly, expression of the membrane-restricted MICB.A2 prevented TRAMP-C2 to form tumors *in vivo* whereas expression of native shedding-sensitive MICB did not (Wu et al., 2009). When the mice were injected with purified sMICB prior to tumor inoculation to imitate the expression of shedding-sensitive MICB, expression of MICB.A2 could not prevent TRAMP-C2 tumor formation. This study provided a proof-of-principle that tumor-specific membrane-bound ligand does not impair NKG2D function *in vivo* and that only the soluble ligand derived from the membrane-bound ligand as a result of shedding induces NKG2D dysfunction to promote tumorigenesis. To provide further evidence supporting this notion, we have created double transgenic TRAMP-MICB and TRAMP-MICB.A2 mice where MICB and MICB.A2 was concurrently expressed with the SV40T oncoprotein in the mouse prostate epithelium directed by the prostate-specific probasin promoter. Sustained immunity was generated by enforced expression of membrane-restricted MICB.A2 to allow long-term tumor-free survival of animals; conversely, enforced expression of shedding-sensitive MICB facilitated bound MIC, is immune suppressive to facilitate tumor progression and metastasis (Wu, unpublished). Together, these studies have suggested that stabilizing membrane-bound NKG2D ligand expression may become valuable avenue for tumor immune therapy.

5.4 Modulation of NKG2D function by tumor microenvironment

Other soluble components than soluble NKG2D ligands in the tumor microenvironment have also been described to facilitate tumors evading NKG2D immunity. One of the widely described factors is TGF-β, which can be secreted by regulatory T cells or tumor cells. TGF-β was well demonstrated to down-regulate of NKG2D expression in Glioma patients (Castriconi et al., 2003; Crane et al., ; Friese et al., 2004). In some cases, TGF-β was also found to inhibit the expression of tumor cell surface NKG2D ligand expression at the transcriptional level (Friese et al., 2004). Indoleamine 2,3-dioxygenase (IDO), a tryptophan (Trp) catabolite, is another well studied component in the tumor microenvironment that may negatively regulate NKG2D function. IDO is generally absent or inactive in cells of the immune system, but it can be induced or activated in macrophages and subsets of dendritic-cell (DC) by specific cytokines, in particular IFN-γ. IDO has also been found in various tumors of different histotypes. Elevated IDO activity was found to be correlated with cancer, such as lung, ovarian, breast cancers, and many other types of malignancies (Ino, 2010; Prendergast et al.). There is evidence that IDO can directly down-regulate NKG2D expression *in vitro* in a time and dose-dependent manner (Song et al. 2010).

6. Interventions to harness NKG2D immunity for cancer treatment

Ample evidence demonstrating that NKG2D function is impaired in cancer patients and that NKG2D dysfunction can facilitate cancer progression to advanced diseases. With the understanding of the mechanisms by which NKG2D function was compromised, in this

section, rationales and optimal strategies to harness NKG2D immunity for potential cancer therapy will be discussed.

6.1 Mechanisms of MIC shedding

Studies have been done in many investigators to understand the mechanisms that regulate MIC shedding for potential therapeutic interventions. A diverse group of enzymes have recently been shown to be involved in MIC shedding. Studies from several groups have shown that inhibition of cellular metalloproteinase activity by GM6001 markedly interferes with MIC shedding. Specific metalloproteinases, such as ADAM (a disintegrin and metalloproteinase)-10 and ADAM-17, were found contributing to MICA shedding (Waldhauer et al., 2008) and ADAM-17 protease was found contributing to MICB shedding (Boutet et al., 2009). The type I membrane MMP (MT1-MMP, also called MMP14) also directly regulates MICA shedding independent of ADAMS (Liu et al., 2010). The thiol isomerase ERp5, which catalyzes disulfide bond formation, reduction, and isomerization, was shown to be required for MIC shedding (Kaiser et al., 2007). This was presumably accomplished by chaperoning conformational alterations of surface MIC through disulphide-bond exchange that render MIC susceptible for proteolytic cleavage.

6.2 Targeting proteases to inhibit MIC shedding

ADAM-10 and -17 and the thioreductase ERp5 have been proposed to be potential cancer therapeutic targets for inhibiting MIC shedding. However, these enzymes are not only involved in pathology of diseases, but also involved in many normal physiological functions. For instance, ADAM-17 is required for generation of the active forms of Epidermal Growth Factor Receptor (EGFR) ligands that is essential for the development of epithelial tissues. In addition, although there are many examples of expression or upregulation of ADAMs in both tumor tissues and cell lines, the precise pattern of their expression within tumors is not always clear (Edwards et al., 2008). Furthermore, targeting ADAM-17 has been in clinical trials with a spectrum of inhibitors for over a decade. However, no single ADAM-17 inhibitor has passed a Phase II clinical trial because of high toxicity and non-specific targeting (DasGupta et al., 2009). As to the possibility of targeting ERp5, it has been suggested that disulfide bond exchange with cell surface molecule to enable the shedding may be a general mechanism by which ERp5 modulates cell signaling (Jordan and Gibbins, 2006). In addition, a wide role of ERp5 in cellular function has been implicated, such as in normal platelet activation (Jordan et al., 2005). These studies suggest that there are many facets of these enzymes that need to be understood before embarking with confidence on targeting them for cancer therapy. Therefore, a more specific and feasible target is needed for inhibiting MIC shedding for cancer therapy.

6.3 Targeting MIC shedding regulatory sequences

By Mass-spectrometry analyses, we and others have shown that MIC is cleaved at multiple sites in the near transmembrane region aa 253-289 in tumor cell lines (Kaiser et al., 2007; Waldhauer et al., 2008; Wang et al., 2009), suggesting that targeting the cleavage site(s) for inhibiting MIC shedding is not therapeutically feasible. Using genetic approach, a dispensable six-aa motif in the α3 ectodomain of MIC (A and B) was identified to be critical for regulating MIC shedding (Wang et al., 2009). Mutation in the six-aa motif completely prevented MIC shedding but did not interfere with MIC to be recognized by NKG2D.

Further study revealed that the six-aa motif is required for MIC to form a physical complex with ERp5, a presumable requirement for MIC to be shed. Due to the "non-invasive" feature of the six-aa motif, molecules or antibodies targeting this six-aa shedding regulatory motif to prevent MIC to interact with ERp5 may be a more feasible therapy.

6.4 Neutralizing sMIC

In a clinical trial with a anti-CTLA-4 antibody blockade or vaccines for melanoma therapy, patients who generated anti-MICA antibodies during the therapy showed significantly better clinical outcome than those who did not (Jinushi et al., 2006). The beneficial effect was shown to act through antibody antagonizing sMICA-induced suppression of NK and CD8 T cell anti-tumor responses. Although not being discussed in this study, the effect of anti-MICA antibody in this particular clinical setting may also be due to elimination of sMIC in the serum and thus elimination of immune suppressive NKG2D$^+$ CD4 T cells. More, anti-MIC antibody has also been shown to sensitive tumor cells to antigen-specific T cells by enhancing DC cross-priming (Groh et al., 2005). Based on these observations, using anti-MIC monoclonal antibody (mAb) to neutralize circulating sMIC and concomitantly to enhance DC cross-priming has been proposed as a cancer therapy. However, clinical implication using anti-MIC antibody must take into consideration that the antibody will also block the interaction of tumor-cell surface MIC with NKG2D and thus block NKG2D-mediated NK cell anti-tumor function. Thus, when applying this approach, it is critical to understand whether NK cell or T cell play a critical role in a particular stage of a specific cancer type. As an alternative approach, phase I clinical trial using adoptively transferred haploidentical NK cells to scavenge plasma sMIC has shown some effect in neuropblastoma patients (Kloess et al. 2010). If donor NK cells are obtainable, this approach may become an effective therapy for many type of cancers.

6.5 Engineering T cells with chimeric NKG2D

A new and very interesting mechanism to utilize the NKG2D-mediated immunity in tumor therapy is expressing chimeric NKG2D-CD3ζ (chNKG2D) in T cells for adoptive cell therapy. By fusing NKG2D with the cytoplasmic signaling domain of CD3ζ chain, NKG2D may induce the anti-tumor activation of T cells independent of TCR signaling, when NKG2D ligand is present on tumor cells. The chNKG2D expressed on NK cells and T cells does not seem to be down-regulated by soluble NKG2D ligand (Zhang et al., 2006; Zhang et al., 2005). This approach had been demonstrated to be very effective in controlling tumor growth in several experimental animal models (Barber et al., 2011; Barber et al., 2008a; Barber et al., 2008b; Zhang et al., 2007). Treatment of mice bearing established ovarian and multiple myeloma with T cells expressing the chNKG2D receptor can lead to long-term, tumor-free survival and induce host memory responses to tumor antigens. This protection is not restricted to the direct effect of chNKG2D-induced activation of T cells upon ligand engagement. Sustained long-term protection against tumors in animal models was found to be achieved through cytokines secreted by the chNKG2D-engineered T cells to induce a proinflammatory environment and re-activate host NK, CD4 and CD8 T cell anti-tumor responses. In ovarian mouse models, adoptive transfer of chNKG2D T cells was found to not only to induce systemic increase in IFNγ, GM-CSF, and perforin but also to eliminate immunosuppressive regulatory CD4 T cells in the tumor microenvironment (Barber and Sentman, 2009; Barber et al., 2008a). Adoptive transferring chNKG2D engineered T cells has

also been shown effective in our tumor models. However, due to the systemic immunoactivation induced by chNKG2D T cells, the long-term safety in clinical application has to be evaluated. chNKG2D-engineered autologous T cells is currently in phase I clinical trial for treating ovarian cancer patients.

7. Conclusion

As emerging evidence demonstrating the significance of sustained NKG2D-NKG2D ligand interaction in anti-tumor responses, in particular solid tumors, it is time to develop therapeutic interventions to harness the NKG2D immunity for anti-tumor therapy. As soluble NKG2D ligands are the culprit for tumor evading NKG2D immunity, interventions to enforce NKG2D-mediated anti-tumor response should be focused on preventing tumor shedding, removal of soluble NKG2D ligand or counteracting the effect of soluble ligand on NKG2D function. More, in the development of tumor vaccines, one should also take into the consideration that across-priming by NKG2D ligand may boost the clinical efficiency of vaccine-induced immune responses. Last but not least, as tumor microenvironment can negatively regulate NKG2D function, co-targeting tumor microenvironment may be necessarily in stratifying NKG2D anti-tumor immunity.

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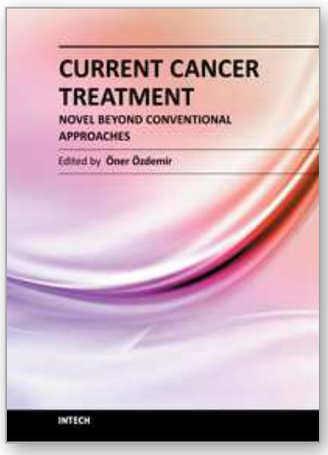

Current Cancer Treatment - Novel Beyond Conventional Approaches

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Currently there have been many armamentaria to be used in cancer treatment. This indeed indicates that the final treatment has not yet been found. It seems this will take a long period of time to achieve. Thus, cancer treatment in general still seems to need new and more effective approaches. The book "Current Cancer Treatment - Novel Beyond Conventional Approaches", consisting of 33 chapters, will help get us physicians as well as patients enlightened with new research and developments in this area. This book is a valuable contribution to this area mentioning various modalities in cancer treatment such as some rare classic treatment approaches: treatment of metastatic liver disease of colorectal origin, radiation treatment of skull and spine chordoma, changing the face of adjuvant therapy for early breast cancer; new therapeutic approaches of old techniques: laser-driven radiation therapy, laser photo-chemotherapy, new approaches targeting androgen receptor and many more emerging techniques.

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InTech Europe
University Campus STeP Ri
Slavka Krautzeka 83/A
51000 Rijeka, Croatia
Phone: +385 (51) 770 447
Fax: +385 (51) 686 166
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InTech China
Unit 405, Office Block, Hotel Equatorial Shanghai
No.65, Yan An Road (West), Shanghai, 200040, China
中国上海市延安西路65号上海国际贵都大饭店办公楼405单元
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