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NKG2D and its ligands in cancer

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NKG2D is an activating immune receptor expressed by NK and effector T cells. Induced expression of NKG2D ligand on tumor cell surface during oncogenic insults renders cancer cells susceptible to immune destruction. In advanced human cancers, tumor cells shed NKG2D ligand to produce an immune soluble form as a means of immune evasion. Soluble NKG2D ligands have been associated with poor clinical prognosis in cancer patients. Harnessing NKG2D pathway is considered a viable avenue in cancer immunotherapy over recent years. In this review, we will discuss the progress and perspectives.

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Introduction

NKG2D is an activating immune receptor which regulates both innate and adoptive immune responses. In human, NKG2D is normally expressed on all NK cells, CD8 T cells, subsets of $\gamma\delta$ T cells and some autoreactive CD4 T cells [1,2]. NKG2D serves as a major activating receptor for NK cells [3]. Activation of NKG2D provides co-stimulatory signal for CD8 T cells [4], NKT cells and in some cases for $\gamma\delta$ T cells.

NKG2D receptor recognizes a broad and structurally diverse range of ligands with differential binding affinities [1,5,6]. Human NKG2D recognizes family of MHC I Chain-related molecules A and B (MICA and MICB, generally termed MIC) and family of six cytomegalovirus

UL16-binding proteins (ULBP1-6) [7,8]. NKG2D ligands are generally absent on the surface of normal cells, but often are induced by tumorigenic insults and are further upregulated by chemotherapy or radiation. Expression of NKG2D ligands on tumor cell surface sensitizes tumor cells to immune cell-mediated destruction by engaging NKG2D to activate NK cells and co-stimulate effector T cells. However, tumor cells frequently escape the immune surveillance of NKG2D pathways by proteolytic-mediated shedding of NKG2D ligands from tumor cell surface or exosome-mediated secretion to release the soluble form of NKG2D ligands [9]. The shed form of NKG2D ligands, sNKG2D-L, has been demonstrated to be highly immune suppressive [2]. High levels of circulating sNKG2D-L were shown to be associated with poor disease prognosis in multiple tumor types, and poor response to immune checkpoint therapy shown in clinical and pre-clinical studies [10*,11**,12–15]. Currently, there are many ongoing efforts to target the NKG2D pathway for cancer immunotherapy. This review will focus on our understanding of the immune suppressive role of soluble NKG2D ligands and ongoing translational perspectives of targeting NKG2D pathway for cancer immunotherapy. We will also discuss the controversies regarding the impact of NKG2D ligands in tumor immunity.

NKG2D signaling

NKG2D-mediated activation signals can override inhibitory signals by NK inhibitory receptors, hence NKG2D is defined as the master activating NK cell receptor [2,16]. NKG2D does not possess any signaling elements within its intracellular domain. NKG2D forms a homodimer and associates with adaptor proteins in its transmembrane domain to a hexameric complex structure and initiate signaling cascades [17,18]. In both humans and mice, stable NKG2D surface expression requires formation of a complex between NKG2D and DNAX-activating protein of 10 kDa (DAP10) [18,19]. In mice, NKG2D can also transiently associate with ITAM (immunoreceptor tyrosine-based activation motif)-containing DAP12, depending on the activation status of the cells [20,21]. Upon ligand engagement, Tyr-X-X-Meth (YXXM) motif within the cytoplasmic domain of DAP10 recruits PI3K and Grb2 to activate NK cell cytotoxicity pathways. DAP12 on the other hand, signals through an immunoreceptor tyrosine-based activation motif which after phosphorylation recruits ZAP70 and Syk to trigger NK cell effector functions. Crystal structure of the NKG2D-NKG2DL complex revealed the rigid adaptation and structural plasticity of NKG2D receptor [22]. Upon binding to its ligands, NKG2D undergoes conformational

changes, resulting in different configurations adopted by identical NKG2D residues in the receptor subunits. This plasticity was thought to explain the ability of NKG2D to recognize such structurally diverse ligands [22,23].

NKG2D ligands in human cancer

The expression of NKG2D ligands is tightly regulated to prevent autoimmune tissue damage. Normal tissues generally do not express NKG2D ligands. Although mRNA or intracellular expression of various NKG2D ligands was detected in some non-tumor tissues, such as gut epithelium and airway epithelium [24,25]. Surface expression of these ligands is rare or only at a threshold level unless under pathogenic insults [24,25]. Among the diversified NKG2D ligands, the MIC family molecules are the best characterized ligands and most prevalently expressed ligands by human tumors, among which MICA is more frequently and abundantly expressed on tumor cell surface than MICB (Table 1). The frequency of surface expression of MICB on tumor cells may depend on the status of HCMV-positivity in individuals, since HCMV protein UL16 was shown to bind and retain MICB in the cytoplasm. [26]. The surface expression of ULBP family members is rather sporadic and less well characterized in human tumors. Orthologs of the MIC family ligands are not identified in rodents whereas homologs of human ULBP family members are expressed by rodents [27–31].

Expression of human NKG2D ligands can be regulated at the transcriptional, post-transcriptional, or post-translational levels. Heat shock stress pathway has been implicated specifically in the regulation of MICA/B [32]. DNA damage has been implicated in the induction of NKG2D ligands via DNA damage response ATM and ATR pathway [33]. Post-transcriptional epigenetic modifications

with histone deacetylase (HDAC) inhibitors along with DNA damaging low-dose chemotherapy or radiotherapy can upregulate MICA/B expression in an array of human tumor cells [34,35**,36–39,40*,41**]. Expression of MICA/B and ULBP3 can also be regulated by endogenous microRNAs [42–44]. At posttranscriptional level, NKG2D ligand expression in tumors is often shed from tumor cell surface to become a soluble form by proteolytic activity or exosomes [9].

High levels of circulating soluble MIC have been associated with poor clinical prognosis and metastasis in multiple cancer types, such as breast, colorectal, ovarian, prostate, lung and other cancers [13,45–47]. Shedding of other family of NKG2D ligands, such as ULBP2 has also been reported to correlate with poor prognosis in selected cancer of solid tumors and hematological malignancies [48–52]. Most recent clinical studies in cohorts of melanoma patients suggested that baseline serum soluble NKG2D ligands may negatively impact clinical outcome of immune checkpoint blockade therapy [10*]. Collectively, these data suggest that circulating soluble NKG2D ligands might be instrumental for cancer prognosis.

The controversy of NKG2D ligands in tumor immunity

The significance of ligand-induced NKG2D activation in controlling tumor initiation or progression was well demonstrated in experimental animal models more than a decade ago [53,54]. Ectopic expression of a NKG2D ligand alone on tumor cells was sufficient to induce tumor rejection, through activation of NK and in some cases CD8 T cells [53,54]. These studies signified the magnitude of ligand-induced activation of NKG2D in controlling tumor. There are ample subsequent studies to support the significance of NKG2D activation in controlling various stages of tumor development [55,56]. Clinically, tumor expression of membrane-bound NKG2D ligands generally correlated with better prognosis [57–59].

Controversy arose over NKG2D ligands in tumor immunity when different experimental model systems were used. In two studies, constitutive expression of membrane-bound NKG2D ligands systemically or in normal epithelium resulted in defects in NKG2D signaling, facilitating increased susceptibility to tumorigenesis [60,61]. On the basis of these observations, an alternative view was proposed indicating that membrane-bound ligands were responsible for the impairment of NKG2D function through chronic stimulation, raising the prospect of negative regulation of tumor immunity by NKG2D ligand expression. Considering the magnitude of NKG2D activation in controlling the survival of NKG2D ligand-positive cells, constitutive expression of NKG2D ligands in normal tissues would demand an inevitable selection process during embryogenesis to tune down NKG2D signaling to avoid NK-cell mediated suicidal signals.

Table 1

NKG2D ligands expression in human cancers

Tumor type	Ligands identified	Known regulation
Carcinoma		
• Ovarian	MICA/B, ULBP2	Shedding
• Cervical cancer	MICA	Shedding
• Breast	MICA/B HER2/3,	Shedding
• Lung	MICA/B	Shedding
• Hepatocellular	MICA/B	Viral, retinoic acid
• Colon	MICA	Shedding
• Renal	MICA, MICB	?
• Prostate	MICA (Hi), ULBP (lo)	Shedding
• Pancreatic	MICA/B	?
• Head and neck cancer	MICA/B	Shedding
Leukemia	MICA/B	Methylation
Lymphoma	MICA, ULBPs	Shedding
Multiple myeloma	MICA	Shedding
Melanoma	MICA, ULBP-2	Shedding
Glioma	MICA/B, ULBP1-3	TGF- β
OsteoSarcoma	MICA	?
Neuroblastoma	MICA, ULBP-2	Releasing soluble form

To resolve this controversy, Liu *et al.* took the advantage of the observation that mouse NKG2D recognizes human MICB and generated two lines of transgenic mouse models, TRAMP/MICB.A2 and TRAMP/MICB. The former expresses a mutated membrane-bound MIC form (MICB.A2) restricted in the prostate tumor in TRAMP mice, whereas MICB in TRAMP/MICB mice can be shed by tumors, resembling those in cancer patients. The TRAMP/MICB.A2 mice enjoyed tumor-free survival although the oncogene was expressed in the prostate epithelium, whereas a percentage of TRAMP/MICB mice developed aggressive prostate tumors [62]. Liu *et al.* further demonstrated that TRAMP/MICB mice with progressive and metastatic tumors all had elevated serum sMICB [62]. The study demonstrated the opposite impact of soluble MIC and membrane-bound MIC on tumor progression in clinically relevant experimental models (Figure 1).

Soluble NKG2D as a cancer immunotherapeutic target

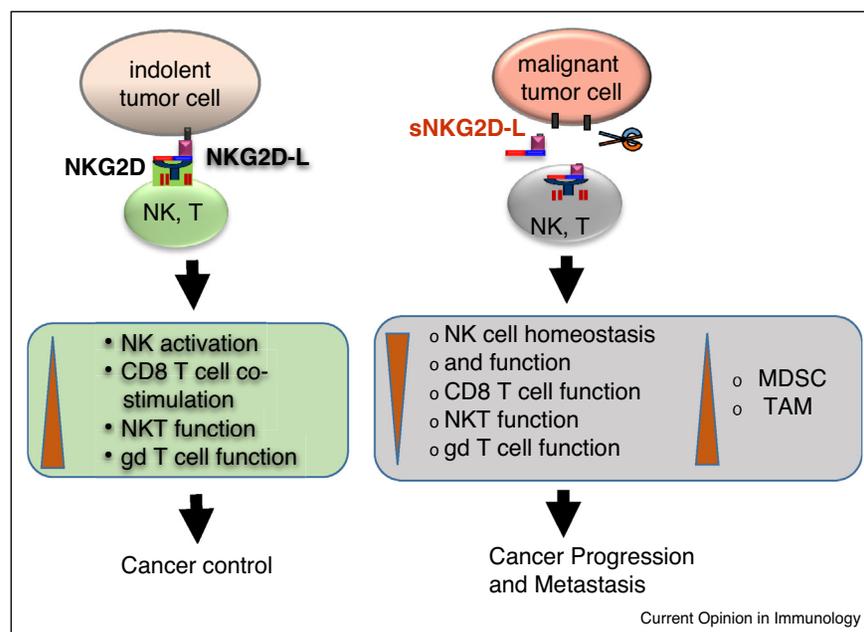
Since MIC is the most expressed NKG2D ligand in human tumors, the impact of sMIC on tumor immunity has therefore been studied the most. sMIC was shown to suppress anti-tumor immune responses through multiple mechanisms, such as down-modulating surface NKG2D expression on effector NK and T cells [47,63], destabilizing the critical CD3/TCR signaling component CD3 ζ on CD8 T cells [64], perturbing NK cell homeostasis [62],

facilitating the expansion of myeloid suppressor cells, and skewing macrophages to alternative tumor-promoting phenotype in the tumor microenvironment [65]. These findings clearly suggested that targeting pathways related to sMIC release or immune suppressive function might be a viable approach for cancer immunotherapy.

Various approaches have been proposed to target sMIC pathway for cancer immunotherapy. These include blocking tumor shedding of MIC through targeting sequences in the $\alpha 3$ domain of MIC that regulates shedding or the thiol isomerase ERp5 that regulates proteinase access to MIC [66,67], removing plasma sMIC through absorption apheresis to precondition patients for adoptive cell therapy [68], and clearing sMIC with monoclonal antibodies [69]. The challenge for pre-clinical validation of these approaches is the requirement for genetically engineered mouse model (GEM) which expresses MIC in tumors, since no MIC homolog is found in rodents.

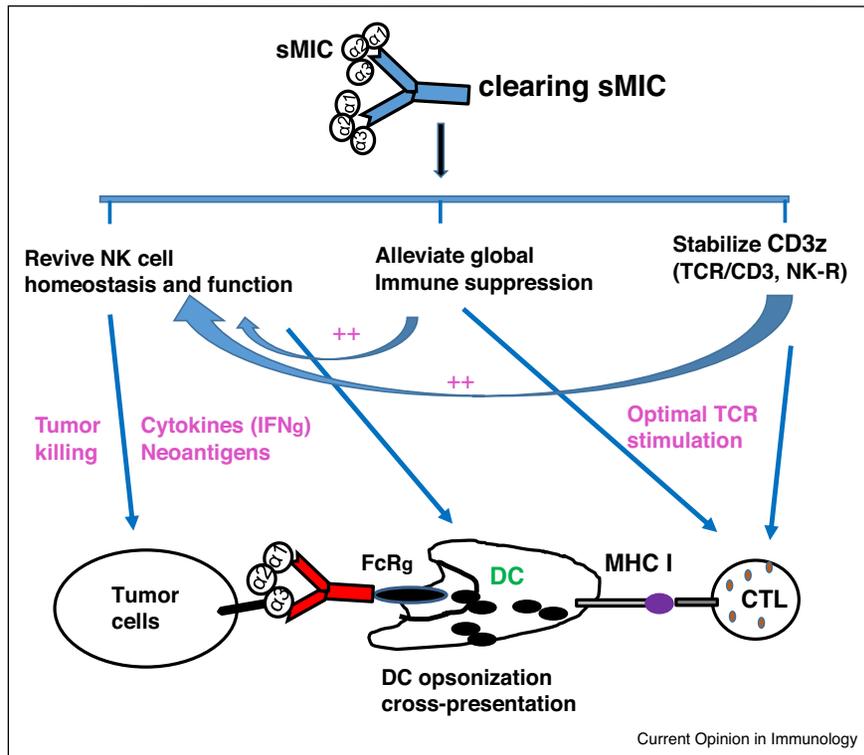
Antibody clearance of sMIC has been tested to be an effective, safe, and feasible therapeutic approach for metastatic cancers. Using the engineered clinically relevant TRAMP/MICB model, Lu *et al.* demonstrated that clearing sMIC with a monoclonal antibody B10G5 significantly reduced primary tumor burden and eliminated metastasis [69]. Mechanistically, B10G5 clearance of sMIC revived and re-invigorated host anti-tumor

Figure 1



Opposite impact of membrane-bound and soluble NKG2D ligands on tumor immunity. Membrane-bound NKG2D ligands (NKG2D-L) stimulate tumor immunity by activating NK cells, co-stimulating CD8 T cell, NKT cell, and subsets of $\gamma\delta$ T cells. Soluble NKG2D ligands (sNKG2D-L) incapacitate tumor immunity through perturbing NK cell hemostasis and function, impairing effector function of CD8 T, NKT, and $\gamma\delta$ T cells, and expanding MDSC and TAMs.

Figure 2



Proposed mechanism of action of clearing sMIC to re-invigorate the anti-tumor responses. Clearance of sMIC was shown to alleviate global immune suppression, restore NK cell anti-tumor immunity, promote DC maturation, and augment antigen-specific CD8 T cell function.

response by a complex mechanism (Figure 2). It was shown that clearing sMIC stabilized NKG2D surface expression on effector cells, restored NK cell homeostasis and function, overcame tumor-induced tolerance of antigen-specific CD8 T cells, and remodeled tumor microenvironment through elimination of MDSC and arginase⁺ macrophages [69^{**}]. Clearing sMIC with B10G5 demonstrated synergistic therapeutic effect with IL-15 agonist ATL-803 and significantly enhanced tumor response to anti-CTLA4 checkpoint blockade therapy in clinically relevant pre-clinical models [11^{**},70].

NKG2D-CAR T cell and NK cell for cancer immunotherapy

NKG2D-CAR-redirectioned T cells and NK cells are currently being explored and under evaluation for safety and efficacy for targeting NKG2D ligand-positive tumors. Variations of NKG2D-CARs have been generated based on the signaling platforms, whether with DAP10 adaptor molecule or with CD28 or 4-1BB signaling platform [71]. All the NKG2D-CARs contain CD3 ζ as the intracellular signaling component. NKG2D-CARs were shown by different research groups to effectively control the growth of a number of tumor types in pre-clinical mouse models, including multiple myeloma [72], ovarian carcinoma [73], and osteosarcoma [74^{*}]. All the NKG2D-CARs presented

effective cytotoxicity against tumor cells, but produced different cytokine profiles with DAP10 signaling from CD28 or 4-1BB signaling platform [71]. NKG2D-CAR-directed NK cells were shown to greatly enhance NK cell activity in general, compared to NK cells with endogenous NKG2D receptor [75]. Toxicity associated with NKG2D-CAR has been observed in preclinical studies [76], but mostly is related to cytokine release syndrome [77]. NKG2D-CAR T cell therapy with DAP10 signaling platform is currently under clinical evaluation for treating metastatic tumors [78].

Concluding remarks

NKG2D-NKG2DL pathway involves multiple effector cell types in controlling tumor progression and can be enhanced by HDAC inhibitors, low dose chemotherapy or irradiation. In patients with metastatic diseases, NKG2D ligands also exist as highly immune suppressive soluble forms in the circulation. Antibody clearance of soluble NKG2D ligands has recently been demonstrated to be an effective and safe method, alone or in combination with immune checkpoint blockade therapy, in pre-clinical models to treat metastatic tumors. NKG2D-CAR-redirectioned T cell and NK cell therapy for metastatic tumors is currently in clinical evaluation for safety and efficacy. It is expected that targeting soluble NKG2D

ligands will be in clinical evaluation in the near future as a single cancer therapy agent or in combination with immune checkpoint blockade therapy. Therapeutic advantage of targeting NKG2D-NKG2DL pathway in combination with HDAC inhibitors or low dose chemotherapy or radiation therapy awaits future investigation.

Conflict of interest

The authors declare that they have no competing interest.

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