Review

Cancer Immunotherapy Using NKG2D and DNAM-1 Systems

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Abstract. *Although tumor antigen-specific immunotherapy, such as dendritic cell vaccine, has recently emerged as a promising clinical approach, one limitation of tumor antigen- and T-cell receptor (TcR)- specific immunotherapy is antigen-specific inhibition by antigen-specific regulatory T-cell and myeloid suppressor cells. Therefore, immunotherapy using a TcR-independent mechanism may be an alternative immunotherapeutic strategy. NKG2D (natural killer, group 2, member D) and DNAX accessory molecule-1 (DNAM-1) are both activated receptors that are strongly expressed on T-cells, γδT-cells, and NK cells. Therefore, the expression of ligands for NKG2D and DNAM-1 on tumor cells plays an important role in tumor opsonization by immune effector cell targeting. Various modulatory methods for up-regulating NKG2D and DNAM-1-ligands have been reported, and included chemotherapeutic agents and hyperthermia. Although there are many obstacles to the utilization of NKG2D and DNAM-1 for cancer therapy, combined treatments using immune cell therapy and chemotherapy that take advantage of NKG2D and DNAM-1 may be an ideal approach.*

Cancer Immunotherapy Using Tumor Opsonization

Cancer immunotherapy aims to activate the immune system for cancer eradication. After a disappointingdly long time, the tide has at last changed due to an understanding of the mechanisms of tumor immunity and success of recent clinical trials (1, 2). Cancer immunotherapy consists of T-cell receptor (TcR)-dependent and -independent mechanisms. Although tumor antigen-specific and TcR-dependent immunotherapy is an ideal immunotherapy, there are many limitations, such as tumor antigen-specific inhibitory mechanisms exerted by regulatory T-cell and myeloid-derived suppressor cells (3-5). Moreover, it is unknown which antigens can be effectively targeted by immune cells for many types of cancer. Alternatively, a biologically therapeutic strategy is to sensitize or opsonize tumor cells and trigger their death using immune cytotoxic effector cells such as activated natural killer (NK) cells and T cells. Recently there have been many studies reporting the clinical effects of nonspecific cytokine-activated T-cells and NK cells. However, cell therapy alone has not achieved sufficiently effective clinical results (6, 7). In this review, we examine the possibility of combining tumor opsonization methods and immune cell therapy focusing on the natural killer, group 2, member D (NKG2D) and DNAX accessory molecule-1 (DNAM-1) systems, and discuss future perspectives on the combination of chemotherapy and immune cell therapy.

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NKG2D and its Ligands

NKG2D is a potent activating receptor expressed on virtually all NK cells, γδT-cells, and CD8 T-cells, and the interaction of NKG2D with its ligands in the tumors plays an important role in the immune response to tumors (8-12). NKG2D is a C-type lectin-like receptor expressed on cell surface and has been classified as a killer cell lectin receptor of superfamily K, member 1 (KLRK1), which is encoded by the *Nkg2d* (*Klrk1*) gene that is located within the NK gene complex (NKC) situated on chromosome 12 in humans (10-12). NKG2D is a homodimer and recognizes a number of stress-induced MHC class I-like ligands. The ligands are summarized and shown in Figure 1. In humans, NKG2D binds to the MHC class I-related proteins MICA and MICB (MHC class I chain-related protein A and B), UL-16 binding proteins (ULBPs). There are six members of the ULBP family of proteins, which are closely related to Rae1 molecules in mice. ULBP1, -2, and -3 and -6 are GPI-

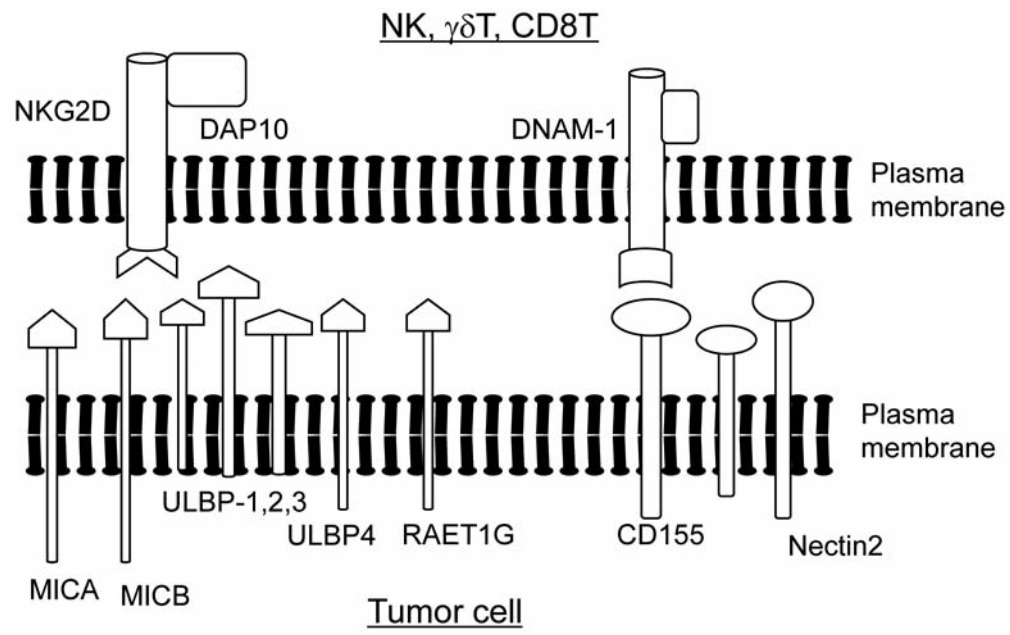

Figure 1. *Multiple ligands for the human NKG2D and DNAM-1 receptors. MICA and MICB have extracellular domains and transmembrane domains. Within the UL16-binding protein (ULBP) family, retinoic acid early transcript 1E (RAET1E) and RAET1G also have transmembrane domains.*

anchored, while ULBP4 and ULBP5 (also known as RAET1E and -G) are transmembrane proteins. These NKG2D ligands are induced in tumor cells by various stresses such as genotoxic stress, DNA-replication inhibitors, heat stress, photodynamic therapy, and chemotherapeutic agents (13-15). There is no inhibitory counterpart known for NKG2D and it is capable of overriding signals provided by inhibitory receptors on NK cells (9, 10, 12). In human, NKG2D associates with the adaptor protein DAP10 that transduces the activation signal of a specific signaling cascade (16). Since DAP10 deficiency results in complete loss of NKG2D signaling in T-cells, it appears that DAP10 is the most important adaptor for NKG2D signaling in these cells (9, 16). Guerra *et al.* (17) reported that NKG2D-deficient mice are defective in tumor surveillance in spontaneous tumor models and this gives rise to aggressive tumors. This indicates that NKG2D plays a critical role in the immunosurveillance of malignancies.

DNAM-1 and its Ligands

Another activating receptor involved in NK- and T cell-mediating tumor cell killing is DNAM-1, a transmembrane glycoprotein constitutively expressed on the majority of T-cells, NK cells, and macrophages. Its ligands are nectin-2 (CD112) and the poliovirus receptor (PVR, CD155), which belong to the nectin/nectin-like family (18) (Figure 1). CD155 is also expressed on epithelial cells, endothelial cells, and antigen presenting cells. CD112 is expressed on epithelial cells. *In vitro* studies have shown that DNAM-1 triggers NK cell-mediated killing of tumor cells expressing CD155 and CD112 (19). DNAM-1 also promotes co-stimulation of CD4 and CD8 T-cells, and mediates adhesion of monocytes to endothelial cells facilitating transendothelial migration (20). It was recently demonstrated that DNAM-1 serves to extend the range of target cells that can activate CD8 T-cell and NK cells and so, may be essential for immune suveillance against tumors and may promoted activation of cytotoxic lymphocytes by nonprofessional antigen-presenting cells (21). It was shown that DNAM-1 mediated NK cell recognition of freshly isolated ovarian carcinoma and neuroblastoma cells (22, 23). In addition, Iguchi-Manaka *et al.* (24) reported that DNAM-1-deficient mice developed significantly more DNAM-1 ligand-expressing tumors than did wild-type mice, which indicates that DNAM-1 plays an important role in immunosurveillance during tumor development.

Immune Evasion by Downregulation of NKG2D Ligands and NKG2D/DNAM-1 Receptors

Tumor cells use multiple mechanisms to bypass NKG2D-mediated killing. Such a mechanism was observed in tumors which often shed soluble NKG2D ligands from their cell surfaces, which can be detected in the blood of cancer patients (25, 26). These soluble ligands can bind to NKG2D

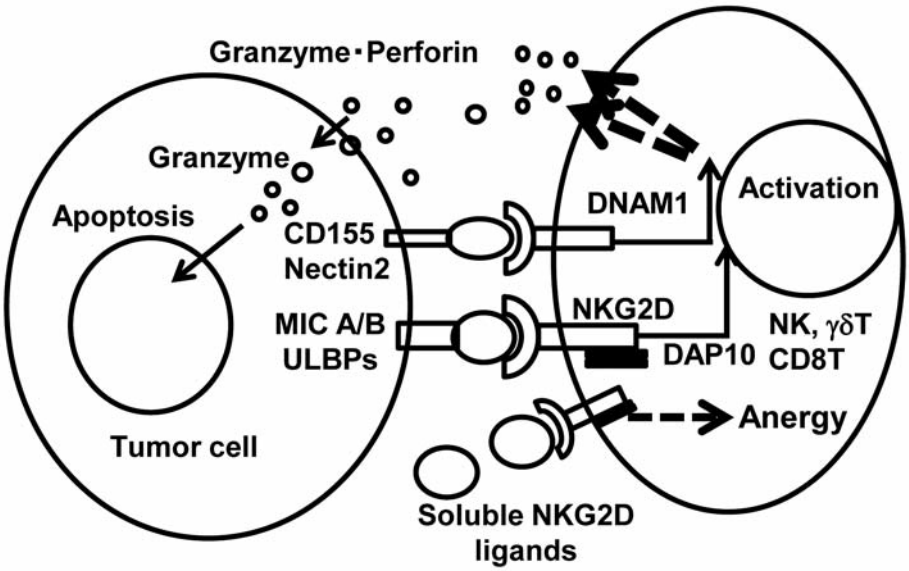

Figure 2. *Interaction between activated T/NK cells and tumor cells through the NKG2D and DNAM-1 systems. After recognition and activation by the NKG2D/DNAM-1 receptor on effector cells for the ligands on tumor cell, cytotoxic molecules such as perforin and granzyme are released and induce apoptosis of tumor cell. The soluble form of NKG2D ligands may occupy the NKG2D receptor and inhibit NKG2D binding to the ligands on tumor cells.*

and downregulate its expression on T-cells and NK-cells, thus effectively anergizing NKG2D-mediated immune recognition (Figure 2). Tumors reduce surface NKG2D ligand expression by shedding the extracellular domain using metalloproteinases or with the assistance of the disulphide-isomerase ERp5 (26). Another important mechanism for NKG2D ligand down-regulation appears to be the production of immunosuppressive cytokines such as transforming growth factor-β, which can be directly excreted by the tumor cells themselves, or by regulatory immune cells that expanded during tumor progression (27, 28). Many studies reported reduced expression of NKG2D or DNAM-1 on NK cells from cancer patients (29-35). For example, Mamessier *et al*. demonstrated that activating receptors such as DNAM-1 and NKG2D on NK cells infiltrating tumor tissue decreased in correlation with NK cell dysfunction throughout breast cancer progression (33).

Contribution of NKG2D Expression on Activated CD8 T-Cells to TcR-independent Cytotoxicity Toward Tumor Cells

Adoptive T-cell transfer therapy, the infusion of *ex vivo* expanded tumor-reactive T cells, resulted in objective tumor responses in patients with melanoma (6). Indeed, expansion of activating $CD8^+$ T-cells with inteuleukin-2 (IL-2) and the agonistic anti-CD3 antibody OKT3 can be easily achieved for adoptive cell therapy. TNK cells, recently designated by Maccalli *et al*. (36), are $NKG2D^+$ $CD8^+$ T-cells, and are relevant T-cell subtypes for immunosurveillance. Negrin's group demonstrated that NKG2D activation can overcome TcR-Class I-restricted cytotoxicity by CD8 T-cells and KIR-inhibition in NK cells (37, 38). Cytokine-activated CD8 T-cells can acquire dual cytotoxic functions: TcR-mediated antigen specific cytotoxicity and TcR-independent NKG2D and DNAM-1-dependent NK-like cytotoxicity against tumor cells (39). It was also shown that cytokine-activated CD8 T-cells induced NK-like cytotoxicity toward tumor cells mainly by NKG2D-ligand interaction (39).

Effects of Chemotherapeutic Agents on Ligands for NKG2D and DNAM-1 (summarized in Table I)

Expression of ligands for NKG2D and DNAM-1 on tumor cells is important for the recognition and killing of tumor cells by effector cells, while shedding of the ligands may inhibit tumor killing by the effector cells (Figure 2). Induced expression of NKG2D ligands on tumors appears to be a promising therapeutic strategy in cancer (9). Heat shock treatment up-regulates MICA on epithelial cells (14, 40). Indeed, the MICA/B promoter contains heat-shock transcriptional elements similar to those found in the promoters of heat shock proteins, such as heat shock protein 70 (41). Moreover, a wide variety of stimuli that causes genotoxic stress and results in DNA replication arrest (summarized in Table I), included histone deacetylase inhibitors, cytarabine, sodium butyrate, sunitinib, retinoic

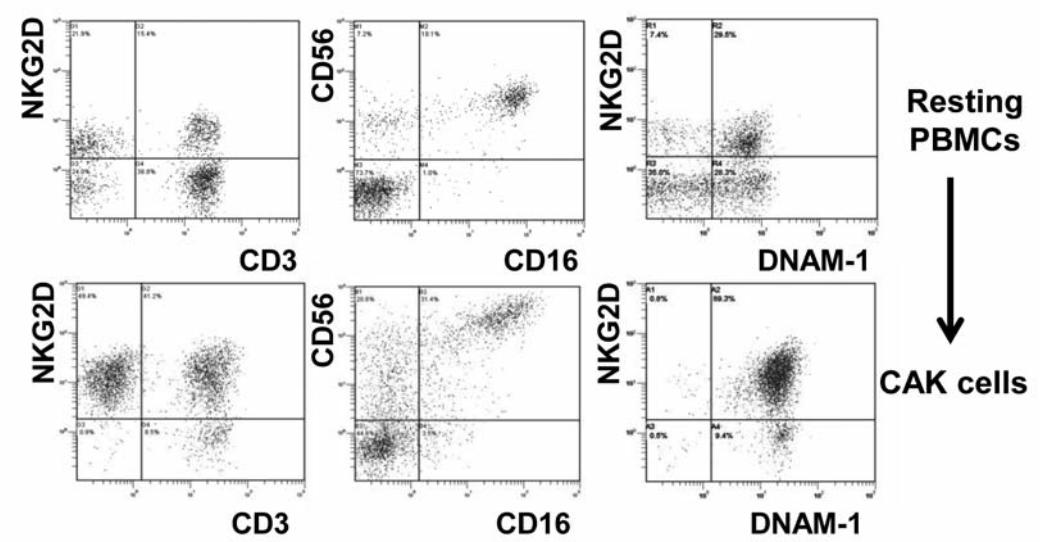

Figure 3. *Increased expression of NKG2D and DNAM-1 on cytokine activated killer (CAK) cells. Peripheral blood mononuclear leukocytes (PBMC) were cultured in the presence of high dose interleukin-2 (IL-2) and the agonistic anti-CD3 antibody OKT3. NKG2D/DNAM-1 expression on mononuclear leukocytes was analyzed by fluorescence activated cell sorting (FACS) analysis as previously described in reference 50. The notable increase in expression of NKG2D and DNAM-1 on CAK cells is shown.*

Table I. *Chemotherapeutic agents.*

Reagents	Cancer cell type	Reference
HDAC inhibitors	Various	47
(Tricostatin A)		47
(FR9011228)		47
Retinoic acid	HCC	49
Sodium valproate	HCC, osteosarcoma	45, 46
Sodium butyrate	Hela, HCC, osteosarcoma	41
Cytarabine	Leukemia cell	42
Sunitinib, sorafenib	Nasopharyngeal carcinoma	48
Bortezomib, MG132	HCC, squamous carcinoma	44,53
Gemcitabine	HCC	50
Hydroxyurea	Leukemia	51
5-Aza-2'-deoxycytidine	Leukemia	43
Hydralazine, valproic acid	Various	54
HSP90 inhibitors	Myeloma	52
Melphalan, doxorbicin	Myeloma	55

HCC: Hepatocellular carcinoma.

acid, gemcitabine, and hydroxyurea (41-55). In our previous study, we demonstrated that gemcitabine induced MICA/B expression at both the protein and mRNA levels in hepatocellular carcinoma cell line HepG2 (50). The DNA damage response pathway, initiated by the ataxia telangiectasia mutated (ATM) or ataxia telangiectasia and the Rad3 related (ATR) kinases, was implicated in the regulation of NKG2D ligands expression in response to these insults. For example, Soriani *et al.* demonstrated that doxorubicin and melphalan up-regulated DNAM-1 and NKG2D ligands on myeloma cells in an ATM-ATR-dependent manner (55). However, in squamous cell carcinoma cells treated with proteasome inhibitors such as bortezomib and M231, the enhanced expression of ULBP-1 was induced by an alternative pathway from ATM/ATR (53).

Modulation of NKG2D and DNAM-1 Systems by Antibody or Inhibitor *via* Ligand Shedding

The NKG2D and DNAM-1 receptor systems can be used as targets for anticancer therapy. Therapeutic MICA-specific antibodies effectively opsonized cancer cells and induced DC-mediated cross-presentation of tumor antigens (56). Bifuntional proteins consisting of a tumor-antigen directed antibody fused to NKG2D ligands effectively coated tumor cells with activating ligands and increased their killing (57, 58). The prevention of MICA/B shedding may also be an important strategy for enhancing cytokine activated killer cell (CAK) cytotoxicity *via* the NKG2D system. There are several reports that have shown the effect of MMP inhibitors on preventing MICA/B shedding by tumor cells (59, 60). Kohga *et al.* demonstrated that a disintegrin and metalloproteinase 9 (ADAM9) are involved in MICA shedding in HCC cells, and sorafenib can modulate ADAM9 expression that resulted in increase of MICA expression (59). Huang *et al.* demonstrated that combined treatment using histone deacetylase inhibitors and metalloproteinase inhibitor caused up-regulation of MICA/B and that inhibition of MICA/B resulted in increased susceptibility to killing by cytokine induced killer lymphocytes (60).

Combinatorial Chemotherapy and Activated Lymphocyte Therapy *via* Enhanced NKG2D and DNAM-1-oriented Systems

Although various kinds of cell-based immunotherapy have been reported, the clinical effects of the therapy alone have been modest (6, 7). Combining immunotherapy with chemotherapy is a promising advancement. Chemotherapy that up-regulates cell surface expression of NKG2D and DNAM-1 ligand and down-regulates shedding of these molecules will need to be effectively combined with cell therapy using activated T-cells and NK cells (60). We previously reported that CAK cells, which consist a heterogenous population composed of activated NK cells and activated killer T-cells, can be induced *in vitro* using peripheral blood lymphocytes treated with high doses of IL-2 and OKT3 (50). We further demonstrated that gemcitabine induces MICA/B expression in hepatocellular carcinoma cells and results in the synergistic enhancement of the cytotoxic effects of CAK cells. Upregulation of NKG2D and DNAM-1 receptors on effector cells can be achieved by *ex vivo* activation with a cytokine such as high dose IL-2 (Figure 3). Thus, combining gemcitabine with CAK cell immunotherapy may have clinical significance in the treatment of various types of cancer. In the near future, harnessing the benefits of immunotherapy using conventional chemotherapy that strengthens the NKG2D/DNAM-1 system will be a promising combinatorial therapy approach.

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