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NKG2D CARs as Cell Therapy for Cancer

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Abstract

The NKG2D cell receptor and its ligands have attracted considerable interest as a potential strategy to attack tumor cells. NKG2D ligands are expressed on most types of tumors, and they demonstrate relative selectivity of ligand expression on tumor cells compared with healthy cells. Several different variants of NKG2D-based chimeric antigen receptors (CAR) have been developed and extensive *in vivo* mechanistic studies performed demonstrating that cytotoxicity and cytokines are important for the efficacy NKG2D CAR adoptive T cell therapy. NKG2D CARs target tumor cells and they also target immunosuppressive cells within the tumor microenvironment. Under certain conditions, NKG2D ligand expression can be found on non-tumor tissue, so potential off-tumor toxicity remain. In this article, we review the use of NKG2D as a basis for CAR targeting of tumors.

Keywords

chimeric antigen receptor; immunotherapy; NK cells; T cells; cytokines; toxicity; immunosuppression; adoptive cell therapy

Introduction

Effector Cells of the immune system have the potential to attack and eradicate cancer cells in patients, but successful cellular therapy requires a sufficient number of tumor-specific effector cells *in vivo*. One approach to achieve this is through the use of chimeric antigen receptors (CAR) that combine specific antigen recognition with signaling capability. CARs can be expressed in lymphocytes, using viral transduction or mRNA transfection, to create a large number of antigen-specific cells that upon binding to antigen-expressing cells mediate cytotoxicity and cytokine production. Enrichment and cell expansion techniques produce $>10^9$ T cells specific for a targeted molecule. A CARs' specificity is often based on antibody scFv regions or TCR binding domains, but NK cell receptors have also been used. These ligand binding domains are combined using recombinant DNA technology with

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extracellular, transmembrane, and signaling domains from other cell proteins, including CD8, CD28, 4-1BB, or OX40. A primary signaling domain is included from CD3 ζ or FcR γ , which induces lymphocyte activation when the CAR binds to its specific ligand^{1,2}. NK cells have the ability to recognize different types of tumor cells through various cell surface receptors. The ligands recognized by NK cell receptors are found on a different tumor types, providing attractive candidates for tumor-targeting. The NK cell receptor NKG2D and its ligands have attracted considerable interest as a potential strategy to attack tumor cells. NKG2D ligands are expressed on most types of tumor cells, and they demonstrate relative selectivity of ligand expression on tumor cells compared with healthy cells³. In this article, we review the use of NKG2D as a basis for CAR targeting of tumors.

NKG2D and its Ligands

There are six to eight NKG2D ligands in humans and mice, with significant differences between ligands within a species. NKG2D ligands in non-human primates are quite different from those in humans, although the extent of cross-reactivity of NKG2D and its ligands among species is not known. Since NKG2D ligands are expressed on various types of tumor cells and immunosuppressive cells (e.g. Tregs and myeloid-derived suppressor cells (MDSCs) within tumor microenvironments, these ligands provide attractive targets for cancer therapy. In fact, most human tumor cells express NKG2D ligands, including: carcinomas (ovarian, bladder, breast, lung, liver, colon, kidney, prostate, melanoma, Ewing's sarcoma, glioma, and neuroblastoma), various leukemias (AML, CML, CLL), lymphomas, and multiple myeloma³. NKG2D ligands can be induced at sites of chronic inflammation, transiently after some infections, and following local irradiation (See Spear, et al.³) for a more detailed review of NKG2D targeting). Thus, NKG2D-based CARs target a large number of tumor types, independent of MHC expression.

A variety of immune cells express NKG2D receptors, including NK cells, NKT cells, $\gamma\delta$ T cells, CD8⁺ T cells, and a subset of CD4⁺ T cells^{4,5}. There are differences between species' expression of the NKG2D receptor. For example, human CD8⁺ T cells constitutively express NKG2D whereas only activated murine CD8⁺ T cells express NKG2D. NKG2D signals differently in NK cells than in T cells. NK cells use either Dap12 (via Syk) or Dap10 (via PI3-kinase) to signal via NKG2D, while T cells only use Dap10⁶. Triggering through NKG2D leads to both cytotoxicity and cytokine release from NK cells. However, Dap10 provides a co-stimulation signal to T cells rather than a primary activation signal.

NKG2D CARs

NKG2D-based CAR therapy was first reported in 2005, and these results demonstrated that an NKG2D CAR was effective in murine models and against human tumor cells^{7,8}. A series of studies from our research group established that NKG2D CAR therapy is effective against a number of tumor types, including multiple myeloma, ovarian carcinoma, and lymphoma *in vitro* and *in vivo*⁹⁻¹². In addition, detailed molecular mechanisms have been elucidated demonstrating that cellular cytotoxicity, cytokine production, and the host immune response are critical for the efficacy of this NKG2D CAR therapy^{9,13-16}. An additional benefit was that the NKG2D CAR not only recognized tumor cells, but the CAR also recognized

NKG2D ligands expressed on immunosuppressive cells, such as MDSCs and Tregs, and endothelial cells within the tumor microenvironment^{13,17,18}. In the past two years, three additional laboratories have used NKG2D based CARs directed against human tumor cells. (Figure 1). In these studies, NKG2D CAR T cells killed tumor cells and produced numerous cytokines in response to human cell lines derived from Ewings sarcoma, ovarian carcinoma, prostate carcinoma, osteosarcoma and a variety of other tumor cell lines. Co-stimulation was provided by Dap10, 4-1BB, or CD28¹⁹⁻²¹.

The full length NKG2D is used in two CAR designs and associates with Dap10 (Figure 1). These CAR designs result in no foreign extracellular domain that can be recognized by the host immune system, in contrast to CARs that involve scFv, TCR, or other fused extracellular proteins. The cytoplasmic portion of CD3z is attached in a reverse orientation because NKG2D is a type II protein. The association with DAP10, which occurs with endogenous NKG2D, provides a co-stimulation signal via PI3-kinase and AKT that promotes Th1-type cytokines and inhibits the production of Th2 cytokines in CD8+ T cells²². Effector cells that express these DAP10 associating CARs produce IFN γ , GM-CSF, various chemokines, and occasionally TNF α , but minimal amounts of IL-5, IL-9, IL-10, or other cytokines^{9,10,19}. The construct used by Chang et al. was expressed in activated NK cells rather than T cells. Even though NK cells normally express NKG2D, these CAR-expressing NK cells demonstrated greater cytotoxicity and produced more cytokines, including IL-13, than mock-transduced NK cells. NK cell function is regulated by positive and negative signals through a variety of activating and inhibitory receptors, respectively⁶. Expression of a CAR in NK cells greatly enhanced NK cells' activity beyond the endogenous NKG2D receptor alone¹⁹.

Two additional NKG2D CAR designs have utilized CD28 or 4-1BB signaling platforms. Because NKG2D is a type II protein, while the other proteins in these constructs are type I proteins, the ligand binding portion of NKG2D (aa 81/82 - 216) was connected to a transmembrane portion of the platform in a reverse orientation that maintained the ligand binding function yet allowed expression as a type I protein^{20,21}. T cells expressing these CARs produced IFN- γ or TNF α upon stimulation with tumor cell lines or primary ovarian cancer specimens. The CAR effector cells were highly cytotoxic, and cytotoxicity was observed using both CD4+ T cells and CD8+ T cells *in vitro*. Analysis of the cytokines produced by these T cells has not been published, so it is possible that these NKG2D CARs produce different cytokines, such as IL-2 or IL-5, compared to the DAP10 associated NKG2D CARs based on the function of CD28 and 4-1BB signaling domains. These cytokines have been observed from T cells using CARs with these signaling domains^{23,24}. Cytokines appear to be a key mechanism used by NKG2D CAR T cells to change the tumor microenvironment and to induce host anti-tumor immunity. Thus, it would be interesting to determine how these different NKG2D CAR designs eliminate tumors *in vivo* and whether differential cytokine production yields unique outcomes in various tumor models and in human cancer.

CD19 CARs that utilize CD28 or 4-1BB costimulatory domains expand greatly *in vivo*. It would be expected that NKG2D CARs that use similar signaling domains will have similar cell expansion *in vivo*. This massive T cell expansion can result in a large percentage of T

cells in the blood expressing the CAR²⁵. In contrast, the NKG2D CAR that associates with DAP10 does not survive long *in vivo* in animal studies¹². CAR down-regulation has been reported, and endogenous NKG2D may be down-regulated in the presence of cytokines or soluble ligands²⁶⁻²⁸. However, NKG2D CAR inhibition does not occur under physiological concentrations of soluble recombinant ligands or patient sera^{8,20}. Membrane bound ligands down-regulated NKG2D on NK cells, but a NKG2D CAR was not down-regulated when it was expressed under the control of a lentiviral promoter but only when the CAR was expressed using mRNA in T cells²⁰. Thus viral transduction of a NKG2D CAR may not be readily inhibited by exposure to soluble ligands or tumor cells that demonstrate high expression of its ligands.

Potential Toxicity Associated with NKG2D – based CARs

NKG2D based CARs have the potential to recognize approximately 90% of human tumor types, but these ligands are also induced under a variety of physiological circumstances which raises concerns about “on-target off-tumor” toxicity. The normal physiologic expression of NKG2D ligands in humans is unknown. Acute exposure to certain microbial components (e.g. LPS) may induce transient ligand expression, although some of these results are based on experimental systems that may not reflect human tissue physiology. Chronic inflammation, such as observed in the joints of patients with rheumatoid arthritis, is associated with expression of NKG2D ligands on synoviocytes²⁹. Activation of DNA repair mechanisms involving ATM/ATR repair pathways induce ligand expression³⁰. Similar mechanisms are likely responsible for the observation that most tumor cells and other cells within the tumor microenvironment express NKG2D ligands. Malignant cells in patients express varying amounts of ligands. For example, tumor cells in patients with advanced cancer demonstrate different amounts of ligand expression compared with patients with limited stages of cancer, or compared with normal individuals^{31,32}. Thus treatments that target NKG2D ligands will need to be used with caution until the extent of ligand expression on normal tissue cells is known. However, large numbers of activated lymphocytes (>10⁹ cells), which express NKG2D and can recognize NKG2D ligand expressing cells (e.g. NKT cells, gd T cells, NK cells), have been infused into patients with little toxicity³³⁻³⁶.

CAR therapies have been developed as cell transplants, and results support the concept that the longer CAR T lymphocytes survive *in vivo*, the better the clinical outcome^{2,37}. CARs based on CD28 or 4-1BB co-stimulation domains result in cell expansion and long-term effector T cell survival *in vivo*, and this is believed to be one reason for the beneficial clinical outcomes reported for CD19-specific CAR therapy. However, NKG2D ligands can be induced on different cell types, so long-lived NKG2D CAR effector cells pose a risk for toxicity against non-tumor cells. CARs recognizing molecules that are not strictly limited to tumor cells can result in significant toxicity, suggesting that maintaining CAR T cells for a long period of time *in vivo* may not be always be an optimal approach to follow³⁸⁻⁴⁰. Toxicity from CAR T cell therapy may be caused by different responses (e.g. cytotoxicity against healthy cells, cytokine storm), but these may be managed and potentially prevented⁴¹. The NKG2D CAR based on the full-length NKG2D protein does not appear to induce long-term CAR T cell survival, which may be a valuable trait to avoid toxicity with these NKG2D CAR cells. Recent evidence indicated that patients demonstrated remarkable

tumor regression within a few weeks following the infusion of CAR T cells, so it may be that long-term persistence of CAR-bearing cells is not required to demonstrate clinical benefits²⁴. Thus, it is possible that administering CAR T cells as “cellular drugs” rather than as cell transplants may be an effective cancer treatment approach for some targets, although this approach may require multiple cellular infusions to demonstrate maximal efficacy.

Why Not Just Use NK Cells?

If NKG2D can be used to target tumor cells and NK cells express high amounts of NKG2D that can trigger cytotoxicity, then why not just infuse NK cells? This is an excellent question without a clear answer. NK cells use NKG2D, among other receptors, to recognize and activate their effector functions in the presence of tumor cells, yet the infusion of a large number of activated NK cells into patients has failed to demonstrate robust clinical responses in many patients^{34,42}. The role and potential of NK cells in cancer therapy is beyond the scope of this review and is reviewed elsewhere⁴³. Clinical data have shown that a large number of NK cells can be given to patients with little toxicity, but the anti-tumor effects have been modest. NK cells express a number of inhibitory receptors that bind to MHC class I and other molecules, and NK cells are sensitive to a variety of inhibitory molecules found within the tumor microenvironment^{6,44}. NK cells may be a useful tool against selected tumor types, but NK cells have not yet provided consistent anti-tumor effects. New approaches that use cytokines to stimulate NK cells, such as IL-15 or IL-18, may result in better efficacy after NK cell infusion. In fact, NK cells' anti-tumor efficacy can be enhanced by transduction of an NKG2D CAR into NK cells¹⁹.

Is It Possible To Induce NKG2D Ligands?

One possible way to improve targeting tumor cells through NKG2D would be to increase the expression of NKG2D ligands on malignant cells or at the tumor site. Localized irradiation induces expression of NKG2D ligands within tumors⁴⁵. In addition, several drugs increase NKG2D ligand expression on tumor cells⁴⁶⁻⁴⁸. Bortezomib (a proteasome inhibitor) increases NKG2D ligand expression on myeloma cells. Histone deacetylase (HDAC) inhibitors increase ligand expression on tumor cells but not on normal blood cells¹⁹. Thus, it is possible that treatment with several medications could be used to increase ligand expression on various tumor cell types, however the induction of ligands on normal cells must be avoided to prevent unwanted toxicity.

Conclusions

NKG2D-based CARs are a novel strategy to target different types of tumors, while inducing potent anti-tumor immunity in patients. The development of CARs based on full-length NKG2D or on the NKG2D ligand binding domain demonstrates the potential to target effector cells against ligand expressing tumor cells. The results indicate that cytotoxicity against tumor cells is important but that cytokines produced by NKG2D CAR T cells are also critical for complete tumor cell eradication and long-term survival in animal models. Unlike many targeting strategies, NKG2D targets tumor cells, immunosuppressive cells, and

other cells in the microenvironment that support tumor survival and progression. This multi-pronged attack on the tumor microenvironment may be one reason that NKG2D targeting is efficacious against several different types of tumors and induces host anti-tumor immunity, even though these CAR T cells do not survive long-term *in vivo*. Because of the potential expression of these ligands on normal cells, there are concerns about potential “off-tumor, on-target” toxicity that must be addressed. There is great potential for NKG2D CARs to improve patient health. The initial clinical results will be vital to demonstrate that the benefits are sufficient and the potential risks manageable.

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NKG2D CARs

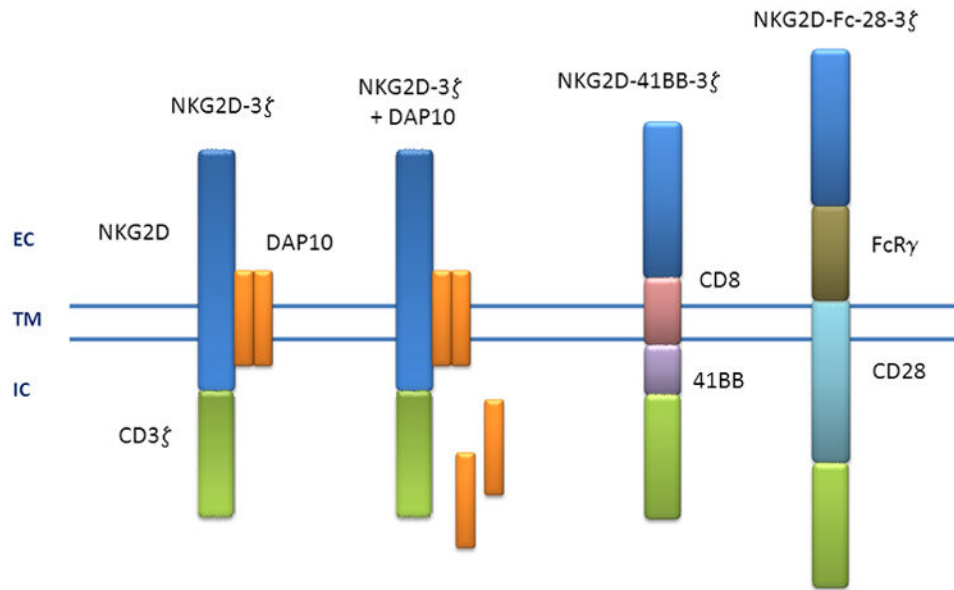


Figure 1. NKG2D based CARs

The different variations of NKG2D based CARs are shown. These CARs are believed to associate as dimers at the cell surface. EC: extra-cellular; TM: transmembrane; IC: intra-cellular. NKG2D-3z (7,8), NKG2D-3z +DAP10 (19), NKG2D-41BB-3z (21), NKG2D-Fc-28-3z (20).