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Chimeric NKG2D receptor-expressing T cells as an immunotherapy for multiple myeloma

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Abstract

Objective—Most myeloma tumor cells from patients express NKG2D ligands. We have reported the development of a chimeric NKG2D receptor (chNKG2D), which consists of the NKG2D receptor fused to the CD3 ζ chain. T cells expressing this receptor kill and produce cytokines in response to NKG2D-ligand+ tumor cells. Therefore we investigated whether human chNKG2D T cells respond against human myeloma cells.

Methods—ChNKG2D T cells were generated from healthy donors and myeloma patients. The effector phase of chNKG2D T cells was analyzed by cell-surface marker expression and human myeloma cell lines were tested for expression of NKG2D ligands. Lysis of myeloma cell lines and cytokine secretion by chNKG2D T cells was determined. ChNKG2D T cells grown in serum-free media, or cryopreserved, were assessed for effector cell functions.

Results—Myeloma cell lines expressed NKG2D ligands. ChNKG2D T cells from healthy donors and myeloma patients lysed myeloma cells, and secreted proinflammatory cytokines when cultured with myeloma cells or patient bone marrow but not with PBMCs or normal bone marrow. Lysis of myeloma cells was dependent on chNKG2D T cell expression of NKG2D and perforin. Additionally, chNKG2D T cells upregulated CD45RO, did not express CD57, and maintained expression of CD27, CD62L, and CCR7, indicating that the T cells were at an early effector stage. Finally, we showed that chNKG2D T cells generated with serum-free media, or when cryopreserved, maintained effector functions.

Conclusion—ChNKG2D T cells respond to human myeloma cells and can be generated using clinically applicable cell culture techniques.

Keywords

Multiple Myeloma; NK cell; Cancer; Chimeric receptors; Immunotherapy

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INTRODUCTION

Multiple myeloma accounts for approximately ten percent of all cases of hematologic cancer in the United States, and despite much research investigating new therapies, the mortality rate of this cancer has not decreased over the past three decades. Many immunotherapies are being investigated in the treatment of myeloma, including cytokines, such as GM-CSF, IFN α , IL-2, or IL-12, and idiotypic vaccination [1–4]. Additional options for immunotherapy are adoptive transfer of T cells that are specific for tumor antigens, or activation, expansion, and transfer of myeloma patient PBMCs [5–7]. While T cell injection has shown promising results in tumors such as melanoma, MHC class I expression is often downregulated on myeloma cells, thus limiting the potential effectiveness of conventional CD8⁺ T cell based therapies [8–10]. However, it has been shown that cytotoxic effector cells derived from myeloma patients may not always require interactions with MHC class I and instead may recognize NKG2D ligands expressed on myeloma cells [5,11].

The ligands for the NKG2D receptor, an activating receptor found primarily on NK cells and CD8⁺ T cells, are expressed on many carcinomas and hematopoietic tumors, such as lymphoma, leukemia, and multiple myeloma [12–17]. In humans, the NKG2D ligands include MICA, MICB, ULBP 1–3, and Letal (ULBP4) [13,14,18]. It has been previously shown that myeloma cells from patients and myeloma cell lines express ligands for NKG2D, including both MICA and the ULBPs [8,19–21]. While NKG2D ligands are not usually expressed on most healthy tissues, ligand expression can be upregulated upon DNA damage, infection, or transformation of cells [13,22,23]. Thus, the NKG2D ligands are a relatively specific tumor cell marker that may serve as a potential target for therapy.

Previously, we created a chimeric NKG2D receptor (chNKG2D), consisting of the NKG2D receptor fused to the CD3 ζ chain [24,25]. When expressed in T cells, chNKG2D T cells lyse NKG2D ligand expressing tumor cells and secrete proinflammatory cytokines and chemokines [24–26]. The anti-tumor response of chNKG2D T cells is dependent upon NKG2D expression on T cells and cell surface NKG2D ligand expression on tumor cells, as chNKG2D T cells do not respond to cells that do not express NKG2D ligands [24–26]. Treatment of mice with established lymphoma or ovarian cancer with chNKG2D T cells did not result in any major side effects [26–28]. In these in vivo experiments, chNKG2D T cell treated mice were allowed to survive 120 days in the lymphoma studies and 325 days in the ovarian cancer studies, and all surviving mice appeared healthy throughout the experiments [27,28]. ChNKG2D T cell responses are also independent of interactions with MHC class I [24,25]. Thus, this immunotherapy can specifically target and destroy NKG2D-ligand expressing tumor cells, while not dependent on MHC class I expression.

In this study, we investigated the possibility of using chNKG2D T cells as adoptive cellular therapy for myeloma. We determined that chNKG2D T cells can be derived from myeloma patient PBMCs, and that these T cells lyse and secrete proinflammatory cytokines when cultured with myeloma cells. Additionally we show that chNKG2D T cells can be generated using methods that can be easily brought to the clinic.

MATERIALS AND METHODS

Tissues and Cell Lines

Blood and bone marrow samples from patients with myeloma or healthy donors were obtained from Dartmouth Medical Center after informed consent. Healthy bone marrow samples were obtained from individuals donating marrow for a related or unrelated transplant. Blood and bone marrow from myeloma patients were on various treatments at the time of blood donation, including newly diagnosed myeloma (n=2), on therapy (n=2), and within two weeks of

autologous PBSC transplant (n=1). Human myeloma cell lines RPMI8226, U266, and MM.1s, and a human EBV-transformed B lymphoblastoid cell line, IM9, were cultured in RPMI supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, 100 µg/ml streptomycin, 1 mM pyruvate, 10 mM Hepes, 0.1 mM non-essential amino acids, and 50 µM 2-mercaptoethanol. MM.1s and IM9 cells were provided by Dr. Terry Landowski (University of Arizona, Tucson, Arizona).

Isolation and transduction of CD8⁺ T cells

Human wtNKG2D and chNKG2D retroviral vectors were made as previously described [25]. Human PBMCs were isolated from healthy donors and myeloma patients as previously described [25]. All procedures were approved by the Internal Review Board at Dartmouth College. PBMCs were stimulated with OKT3 (OKT3, 40 ng/ml) and 50 U/ml rHuIL-2 for three days. Retroviral transduction of PBMCs was performed as previously described [25]. Two days after transduction, T cells were selected in complete RPMI media containing G418 (0.5 mg/ml) plus 50 U/ml rHuIL-2 for an additional 3 days. CD8⁺ cells were isolated using MACS columns and were cultured for an additional 2 days in 50 U/ml rHuIL-2 before use [25]. For serum-free media comparison studies, cells were grown in complete RPMI media, AIM V media (Invitrogen, Carlsbad, CA), Stemline media (Sigma, St. Louis, MO), or X-Vivo 15 media (Cambrex, Walkersville, MD) from the time of PBMC isolation until use in effector assays. For T cell cryopreservation, wtNKG2D and chNKG2D T cells were cryopreserved at 10⁷ cells/ml in 10% DMSO and 90% heat-inactivated fetal bovine serum, and thawed one week later for use in effector assays.

RT-PCR

Total RNA from tumor cell lines was extracted and specific primers were used for amplification of the human NKG2D ligand sequences as previously described [26]. The PCR products were analyzed using agarose gels and visualized by staining with SYBR Safe (Invitrogen, Carlsbad, CA).

Flow cytometry

For flow cytometric analysis of NKG2D ligand expression, tumor cells were incubated with human FcR blocking reagent (Cohn's fraction) (Sigma, St. Louis, MO), stained with human NKG2D-mouse IgG1 fusion protein or mouse IgG1 isotype control, followed by staining with PE-labeled rat anti-mouse IgG1 (A85-1, BD Pharmingen, San Diego, CA). For analysis of T cell differentiation markers, cells were incubated with Cohn's fraction, and stained with PE-conjugated anti-CD3 (Beckman Coulter, Fullerton, CA), APC-conjugated anti-CD8 (Invitrogen), and either FITC-conjugated anti-CD57 (Invitrogen), anti-CD27, anti-CD45RO, anti-CD62L (all from ebioscience, San Diego, CA), or anti-CD62L (R&D Systems, Minneapolis, MN). All isotype controls were purchased from ebiosciences. Cell fluorescence was monitored using a FACSCaliber cytometer (Becton Dickinson, San Jose, CA).

Cytotoxicity by gene-modified T cells

Lysis of myeloma target cells was determined by 5-hr ⁵¹Cr release assays as previously described [25]. To block NKG2D receptors, T cells were pre-incubated for two hours at 37°C with anti-NKG2D antibodies (clone: 1D11, 20µg/ml, sodium azide free) or isotype control antibodies prior to addition of target cells [26]. To inhibit the perforin pathway, T cells were preincubated at 37°C with 20 nM concanamycin A (CMA, Sigma, Sigma, St. Louis, MO) or EtOH vehicle for one hour before addition of target cells.

Cytokine production by transduced T cells

Culture of irradiated myeloma cells (10^5 cells, 120Gys), autologous PBMC, or bone marrow cells with gene-modified T cells (10^5) was performed in 96-well plates. Cell-free conditioned medium (CM) were collected after 24 and 72 hr. Twenty-four-hour CM were assayed for IFN γ by ELISA using human DuoSet ELISA kits (R&D Systems). Seventy-two-hour CM were used for detection of other cytokines and chemokines using Bio-Plex kits (BioRad, Hercules, CA) based on the manufacturer's protocol. Bio-Plex analysis was performed by the Immune Monitoring Laboratory of the Norris Cotton Cancer Center (Lebanon, NH)

Statistical analysis

Differences between groups were analyzed using the Student's t test. Values of $p < 0.05$ were considered significant.

RESULTS

Human myeloma and B lymphoblastoid cell lines express NKG2D ligands

Almost all primary human myeloma samples express ligands for NKG2D, including MICA and ULBPs [8,19–21]. To confirm whether human myeloma cell lines RPMI8226, U266, and MM.1s express NKG2D ligands on their cell surfaces, these cells were stained with a soluble NKG2D receptor, consisting of the NKG2D receptor fused to mouse IgG (Figure 1A). We also stained the IM9 cell line, an EBV-transformed B lymphoblastoid cell line derived from a myeloma patient, to test a B cell line in an earlier stage of B cell differentiation than myeloma cells. All three myeloma cell lines and the lymphoblastoid cell line were positive for cell surface NKG2D ligand expression. To determine which of the NKG2D ligands these cells expressed, RT-PCR was performed for each ligand. RPMI8226, U266, and IM9 cell lines expressed MICA, MICB, and Letal. MM.1s cells expressed MICA and MICB but did not express Letal. U266 cells also expressed low levels of ULBP1, while no ULBP2 or ULBP3 expression was detected on any of the cell lines (data not shown).

ChNKG2D T cells express early effector differentiation markers

T cells that have been activated and expanded for more than two weeks using standard expansion protocols are at a late effector stage. While these cells demonstrate more robust effector responses *in vitro*, they display reduced efficacy *in vivo* [9,29–31]. T cells that are in an early effector differentiation state may be more effective *in vivo*. Consequently, we wanted to determine the differentiation state of chNKG2D T cells to ensure they would provide the maximum potential *in vivo* response. ChNKG2D T cells were stained with antibodies specific for the T cell differentiation markers CD57, CD27, CD45RO, CCR7, and CD62L after ten days of culture (Figure 1B), the timepoint when *in vitro* assays are performed and *in vivo* transfer would likely occur. ChNKG2D T cells expressed little CD57, but increased expression of CD45RO. They maintained expression of the costimulation molecule CD27, chemokine receptor CCR7, and L-selectin CD62L. ChNKG2D T cells generated from myeloma patients had similar phenotypes, with low CD57 expression, increased CD45RO expression, and sustained CD27, CCR7, and CD62L expression (data not shown). The expression pattern of these cell surface markers indicates that chNKG2D T cells prepared from healthy donors and myeloma patients are at an early effector differentiation stage, suggesting they may demonstrate clinical efficacy.

ChNKG2D T cells lyse myeloma cells in an NKG2D- and perforin- dependent manner

Since all cell lines were positive for NKG2D ligand expression, we tested whether chNKG2D T cells could lyse myeloma cells. ChNKG2D T cells derived from healthy donors significantly killed IM9, MM.1s, RPMI8226, and U266 cells, compared to T cells expressing wtNKG2D at

all E:T ratios tested (Figure 2). Additionally, when NKG2D was blocked with anti-NKG2D antibodies, chNKG2D T cells no longer killed RPMI8226 or U266 cells (Figure 2 C–D). These results indicate that chNKG2D lysis of myeloma cells was dependent on NKG2D.

CD8⁺ T cells can kill myeloma cells using multiple mechanisms, including the perforin pathway [32,33]. We have previously shown that murine chNKG2D T cells use perforin and/or FasL to lyse murine lymphoma and ovarian cancer cells [27,28]. To determine if chNKG2D T cell derived perforin was necessary to kill human myeloma cells, wtNKG2D and chNKG2D T cells were incubated with concanamycin A (CMA), an inhibitor of perforin dependent killing, prior to addition of myeloma cells (Figure 2 E–F). Incubation of chNKG2D T with CMA abolished their ability to lyse myeloma cells, demonstrating that chNKG2D T cell derived perforin was essential for killing of human myeloma cell lines. A similar inhibition of killing was also seen when CMA-treated chNKG2D T cells were cultured with MM.1s and IM9 cells (data not shown). Therefore, chNKG2D T cells lysed human myeloma and lymphoblastoid cells in an NKG2D- and perforin- dependent manner.

ChNKG2D T cells derived from myeloma patients lyse and secrete proinflammatory cytokines in response to myeloma cell lines

The data in Figure 2 were generated with T cells derived from healthy donors. As T cells used in a clinical trial would be derived from myeloma patient blood, we wanted to assess the extent that chNKG2D T cells from myeloma patients kill myeloma cells. PBMCs were obtained from five patients with multiple myeloma. These patients were at various stages of disease and treatment phases at the time of blood donation, including patients with newly diagnosed myeloma, patients currently on therapy, and patients within two weeks of autologous PBSC transplant. ChNKG2D T cells were successfully generated from all five myeloma patient blood samples, and these chNKG2D T cells specifically lysed myeloma cell lines and the B cell lymphoblastoid cell line (Figure 3).

Because cytokine production by chNKG2D T cells is an essential mechanism for tumor elimination in two murine cancer models [27,28], wtNKG2D and chNKG2D T cells derived from myeloma patients were cultured with tumor cells and the secretion of proinflammatory cytokines and chemokines was determined (Figure 4). Compared to wtNKG2D T cells, chNKG2D T cells derived from myeloma patients secreted significant amounts of proinflammatory cytokines IFN γ , GM-CSF, and TNF α , and the chemokine CCL5. Although wtNKG2D T cells from the myeloma patients were activated, transduced, and expanded in the same manner as chNKG2D T cells, the wtNKG2D T cells did not lyse human myeloma cell lines nor did they secrete high amounts of cytokines. This indicates that the CD8⁺ T cells in the myeloma patients' blood were not able to respond to the myeloma cell lines even upon activation, but that expression of the chNKG2D receptor provided a unique means for the myeloma patients' T cells to recognize and respond against the myeloma cells.

ChNKG2D T cells secrete IFN γ when cultured with myeloma bone marrow but not with healthy PBMC or bone marrow cells

To address whether chNKG2D T cells respond to primary patient or normal bone marrow cells, T cells transduced with wtNKG2D or chNKG2D were cultured with myeloma cell lines, bone marrow from myeloma patients, normal bone marrow, or autologous PBMCs (Figure 5A). ChNKG2D T cells secreted IFN γ when cultured with myeloma cell lines RPMI8226 or U266 and with primary bone marrow from myeloma patients, there was no significant IFN γ secretion when chNKG2D T cells were cultured with autologous PBMCs or healthy bone marrow cells. WtNKG2D T cells did not secrete IFN γ demonstrating the requirement for the chimeric receptor. As myeloma bone marrow samples often contain low amounts of tumor cells, we wanted to determine whether chNKG2D T cells could respond to low numbers of NKG2D-

ligand positive cells mixed among normal cells. Autologous PBMCs from a healthy donor or healthy bone marrow cells were spiked with 0.2%, 1%, or 5% RPMI8226 cells (Figure 5B). ChNKG2D T cells secreted IFN γ when cultured with 0.2% NKG2D-ligand positive cells, and they responded in a dose dependent manner, secreting more IFN γ as the number of tumor cells increased. These data indicate that chNKG2D T cells are able to respond to primary myeloma bone marrow samples but they do not respond to healthy bone marrow or autologous PBMCs.

ChNKG2D T cells cultured in serum-free media have similar anti-tumor efficacy as T cells grown in serum-containing media

To develop the use of chNKG2D T cells as adoptive cellular therapy for multiple myeloma, we assessed whether chNKG2D T cells could be generated in serum-free media. ChNKG2D T cells were activated and expanded in RPMI with 10% FCS, as performed in the previously described experiments, or in three serum-free medias: AIM V, Stemline, or X-Vivo 15 (Figure 6). The same numbers of PBMCs were activated with OKT3 and IL-2 in each media, and the T cell expansion was evaluated. While the T cells that were grown in serum-containing media demonstrated high expansion and survival, chNKG2D T cells were able to survive and proliferate in each of the serum-free media. One striking difference was that cells grown in Stemline serum-free media expanded and/or survived significantly better than those grown in AIM V or X-Vivo 15 media. When comparing the function of chNKG2D T cells grown in serum-containing and serum-free media, chNKG2D T cells cultured with or without serum had similar effector functions. ChNKG2D T cells cultured in Stemline media lysed RPMI8226 cells as well as chNKG2D T cells grown in serum-containing media. In addition, chNKG2D T cells maintained in all three serum-free media secreted similar levels of IFN γ when cultured with myeloma and lymphoblastoid cells (Figure 6). Therefore chNKG2D T cells can be generated and maintained in serum-free media. While the type of media did not alter the T cell effector functions, the formulation of serum-free media did affect the final cell yield.

Cryopreserved chNKG2D T cells maintain anti-tumor activity

Another aspect of producing cells for clinical trials is the need to cryopreserve cells before infusion into patients. Consequently, to ensure that chNKG2D T cells would maintain anti-tumor functions after cryopreservation, chNKG2D T cells were cryopreserved after ten days of culture. A week later, the cells were thawed and used in effector assays measuring lytic function and IFN γ secretion (Figure 7). Cryopreserved chNKG2D T cells lysed RPMI8226 cells as well as fresh (non-cryopreserved) chNKG2D T cells. Furthermore, both fresh and cryopreserved chNKG2D T cells secreted similar amounts of IFN γ when cultured with myeloma and lymphoblastoid cell lines. Overall, these proof-of-principle studies confirm that chNKG2D T cells maintain anti-myeloma effector functions even when activated and expanded in serum-free media and after cryopreservation.

DISCUSSION

Since most human multiple myeloma cells express NKG2D ligands, we sought to determine if chNKG2D T cells could recognize and lyse human myeloma cells. ChNKG2D T cells derived from both healthy donors and myeloma patients killed myeloma cells and secreted proinflammatory cytokines when cultured with myeloma cells and primary myeloma bone marrow samples, but not when cultured with normal cells. Additionally, chNKG2D T cells could be developed with a method that can be potentially brought to the clinic, using OKT3 and IL-2 activation, serum-free media, and cryopreservation. Thus it is feasible to use chNKG2D T cells derived from multiple myeloma patients as adoptive immunotherapy to recognize and lyse human myeloma cells.

A myeloma patient's endogenous T cells and NK cells may recognize and respond to myeloma cells. However, tumor cells utilize many mechanisms to escape immunosurveillance, thus avoiding recognition and lysis by immune cells. Myeloma cells may have a decreased MHC class I expression, thus avoiding recognition by CD8⁺ T cells [8]. On the other hand, decreased MHC expression may make the myeloma cells more susceptible to recognition by NK cells, because MHC class I molecules are ligands for inhibitory receptors on NK cells. Conversely, it has been shown that tumor cells can shed cell surface molecules in a soluble form. Tumor cells, including myeloma cells, may release a soluble form of MICA, and this has been shown to inhibit endogenous NK and T cell responses to tumors by downregulating NKG2D receptor expression [16,34–37]. Although this could potentially inhibit chNKG2D T cells, we have previously shown that chNKG2D T cell activity was only inhibited at super-physiological concentrations of soluble MICA ($>1.5 \times 10^6$ pg/ml) [25]. A recent study in multiple myeloma patients found the mean sMICA level in 97 myeloma patients to be 429 pg/ml, a level far below the amount that caused chNKG2D T cell inhibition [34].

ChNKG2D T cells derived from myeloma patients lysed myeloma cell lines and secreted proinflammatory cytokines and chemokines. While there is evidence that myeloma patient T cells have the potential to recognize myeloma tumor antigens, wtNKG2D expressing T cells derived from the patients that were activated and expanded in the same manner did not respond to the myeloma cell lines [7]. These results indicate that these patient T cells could not recognize these myeloma cells without expression of the chNKG2D receptor. One possible reason for this could be due to a MHC mismatch, since these tumor cell lines are allogeneic to the patient T cells, while chNKG2D T cells do not require MHC class I interactions. Additionally the patients were receiving various treatments at the time of blood donation, and some of these therapies may inhibit endogenous immune responses. However, two of the patients analyzed were newly diagnosed and had not yet received therapy. Another possibility is that the T cells derived from the myeloma patients were somehow suppressed, exhausted, or tolerant to myeloma tumor antigens, however addition of the chNKG2D receptor circumvented any suppression and allowed the patients' T cells to recognize myeloma cells.

ChNKG2D T cells secreted proinflammatory cytokines IFN γ , GM-CSF, and TNF α during coculture with myeloma cells. IFN γ can have multiple anti-tumor effects including increasing MHC class I expression, anti-proliferative effects on the tumor cells, and may play a role in increasing antigen presentation [38–41]. IFN γ also upregulates the expression of TRAIL and leads to increased apoptosis of myeloma cells [42]. GM-CSF is often included in clinical trials for the treatment of myeloma and may play a role in increasing tumor antigen presentation and regeneration of host immune cells after chemotherapy and stem cell transplantation [2,3,43, 44]. In mouse models of lymphoma and ovarian cancer, chNKG2D T cell-derived IFN γ and GM-CSF were critical for anti-tumor efficacy *in vivo* [27,28]. Thus chNKG2D T cells may not only directly kill myeloma cells, but may also increase the susceptibility to apoptosis, recognition by the autologous immune system, and the development of host immune response due to increased antigen presentation.

CD8⁺ T cells use multiple mechanisms to kill tumor cells, including the perforin pathway and Fas/FasL interactions. There is evidence that CD8⁺ T cells from myeloma patients use perforin to kill myeloma cells [32,45]. Previous work has shown that murine chNKG2D T cells lyse murine lymphoma cells, RMA, using both perforin and FasL pathways, while they lyse an ovarian cell line, ID8, using the only perforin pathway [27,28]. Using concanamycin A to block the release of perforin, we showed that human chNKG2D T cells required perforin to lyse myeloma cells. Myeloma cells are often resistant to killing by the Fas/FasL pathway, but treatment with IFN γ or IFN α can upregulate Fas-induced apoptosis by increasing Fas expression [46–48].

Evidence from both mouse models and clinical studies have shown that the differentiation state of adoptively transferred T cells affects the anti-tumor capabilities *in vivo* [9,29–31]. Many current protocols for generating T cells for clinical trials involve multiple rounds of activation and long-term expansion prior to adoptive transfer [9,10]. Such protocols induce T cells to differentiate to a late effector state, with decreased expression of costimulation molecules, decreased proliferation potential, shorter telomere lengths, decreased cytokine receptor expression, and decreased ability to home to secondary lymphoid structures [9,29]. As a result, these T cells are less efficacious *in vivo* despite having robust responses *in vitro*. It was important that we determine what effector state chNKG2D T cells were in after transduction to ensure they would potentially have the maximum effect *in vivo*. ChNKG2D T cells were only cultured for ten days, a relatively short time period compared to T cell expansion protocols currently used for clinical trials [9,10]. Analysis of multiple differentiation markers indicated that chNKG2D T cells were at an early effector phase that is associated with *in vivo* efficacy.

Previous studies with human chNKG2D T cells used PHA to stimulate T cell proliferation, and serum-containing media to grow T cells, however these cell culture methods would not be acceptable for clinical trials [25,26]. This study illustrated that chNKG2D T cells can be generated using clinical grade OKT3 and IL-2, serum-free media, and maintain effector functions after cryopreservation. Moreover, chNKG2D T cells could be generated from myeloma patients and responded to primary myeloma samples. We also did not observe any major side effects in previous studies treating lymphoma or ovarian tumor bearing-mice with chNKG2D T cells [24,26–28]. Overall this proof-of-principle study developed clinically applicable methods for generation and maintenance of chNKG2D T cells and showed potential applications as a novel therapy for multiple myeloma.

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Figure 1. Surface expression of NKG2D ligands on human myeloma and lymphoblastoid cell lines and differentiation markers on chNKG2D T cells

(A) Analyses using a soluble human NKG2D-mouseIgG1 fusion protein (filled histogram) or mouse IgG1 isotype control (open histogram) and a PE-anti-mouse IgG1 antisera was performed on RPMI8226, U266, MM.1s, or IM9 cells. (B) Ten days after stimulation with OKT3 and IL-2 and subsequent retroviral transduction, chNKG2D T cells from a healthy donor were analyzed for T cell differentiation markers (filled histograms) or isotype controls (open histograms). CD57, CD27, CD45 RO, CCR7, and CD62L expression are shown. Histograms are representative of data from two healthy donors.

Figure 2. ChNKG2D T cells lyse myeloma and lymphoblastoid cell lines in an NKG2D and perforin dependent manner

T cells from healthy donors were transduced with wtNKG2D (triangles) or chNKG2D (squares) and were used as effector cells in ^{51}Cr release assays against (A) IM9, (B) MM.1s, (C,E) RPMI8226, or (D,F) U266 cells at the indicated E:T ratios (1:1, 5:1, or 25:1). ChNKG2D transduced T cells had higher specific lysis at all E:T ratios compared to wtNKG2D T cells, ($p < 0.05$). (C and D) WtNKG2D and chNKG2D T cells were incubated with anti-NKG2D Abs (open symbols) or with isotype control IgG Abs (closed symbols) before addition to tumor cells. Blocking NKG2D significantly reduced the cytotoxicity of chNKG2D-transduced T cells against tumor cells at all E:T ratios compared with isotype control ($P < 0.05$). (E and F)

WtNKG2D and chNKG2D T cells were pre-treated with concanamycin A (CMA, open symbols) or EtOH vehicle (closed symbols) before addition of tumor cells. Inhibiting the perforin pathway by CMA significantly reduced the cytotoxicity of chNKG2D-transduced T cells against tumor cells at all E:T ratios compared with control ($P<0.05$). Data are represented as mean + SD of triplicates and are representative of 2 to 5 separate experiments.

Figure 3. ChNKG2D-transduced T cells from myeloma patients kill myeloma and lymphoblastoid cell lines

T cells from myeloma patients were transduced with wtNKG2D (open squares) or chNKG2D (closed squares) and used as effector cells in ^{51}Cr release assays against (A) RPMI8226, (B) U266, (C) MM.1s, or (D) IM9 at the indicated E:T ratios (1:1, 5:1, 25:1). T cells from up to five different patients were tested on different days, with each line connecting the data points from a patient. ChNKG2D transduced T cells from myeloma patients had higher lysis compared to wtNKG2D T cells, ($p < 0.05$). Data are represented as mean + SD.

Figure 4. Myeloma patient-derived chNKG2D T cells secrete proinflammatory cytokines and chemokines when cultured with myeloma cell lines

WtNKG2D-(open bars) and chNKG2D-transduced T cells (closed bars) from myeloma patients were cultured for 24 or 72 hours with RPMI 8226 cells, U266 cells, or media alone as indicated. Tumor cells were also cultured alone (hashed bars). Cell-free CM were analyzed for cytokines (IFN γ , GM-CSF, and TNF α), and the chemokine CCL5. Data are presented as mean + SD and are representative of three different myeloma patient samples and one healthy donor. When cultured with tumor cells, chNKG2D T cells produced more cytokines than wtNKG2D T cells or tumor cells cultured alone (p<0.001).

Figure 5. ChNKG2D T cells secrete IFN γ when cultured with myeloma bone marrow but not with healthy PBMC or bone marrow cells

WtNKG2D (open bars) and chNKG2D T cells (closed bars) generated from healthy donors were cultured for 24 hours with (A) RPMI 8226 cells, U266 cells, autologous PBMCs (auto PBMC), healthy bone marrow (healthy BM), myeloma patient bone marrow cells (Pt A BM, Pt B BM), or media alone. (B) The wtNKG2D (open bars) and chNKG2D T cells (closed bars) were cultured with autologous PBMCs or healthy bone marrow spiked with 0.2%, 1%, or 5% RPMI8226 cells, as indicated. Tumor cells were also cultured alone (hashed bars). Cell-free CM was analyzed for IFN γ . Data are presented as mean + SD and are representative of T cells from two different healthy donors. An * indicates differences between wtNKG2D and chNKG2D samples ($p < 0.001$).

Figure 6. ChNKG2D T cells cultured in serum-free media have similar anti-tumor efficacy as T cells grown in serum-containing media

(A) ChNKG2D T cells from a healthy donor were cultured in serum-containing media (RPMI-10%FCS, ■) or serum-free media, either AIM V (◆), Stemline (▲), or X-Vivo 15 (X). The number of cells obtained with each media was counted over the course of the cell culture until the time of CD8⁺ T cell separation on day 8. (B) WtNKG2D (open squares) T cells and chNKG2D T cells (closed squares) cultured in RPMI-10% FCS and chNKG2D T cells cultured in Stemline medium (closed triangles) were used as effector cells in ⁵¹Cr release assays against RPMI8226 cells at the indicated E:T ratios (1:1, 5:1, or 25:1). ChNKG2D transduced T cells cultured in serum-containing and serum-free media had higher specific lysis at all E:T ratios

compared to wtNKG2D T cells ($p < 0.05$). (C) WtNKG2D (wt) and chNKG2D (ch) transduced T cells cultured in serum-containing media or serum-free media were cultured with myeloma cell lines for 24 hours, and cell-free CM were analyzed for $\text{IFN}\gamma$. Data are presented as mean + SD and are representative of 2 separate experiments. When cultured with tumor cells, chNKG2D T cells cultured in both serum-containing and serum-free media produced more $\text{IFN}\gamma$ than wtNKG2D T cells ($p < 0.05$).

Figure 7. Cryopreserved chNKG2D T cells maintain anti-tumor activity

(A) WtNKG2D (open symbols) and chNKG2D T cells (closed symbols) that were used fresh (squares) or that were cryopreserved and thawed one week later (triangles) were used as effector cells in ^{51}Cr release assays against RPMI8226 cells at the indicated E:T ratios (1:1, 5:1, or 25:1). Both fresh and cryopreserved chNKG2D T cells had higher specific lysis at all E:T ratios compared to wtNKG2D T cells, ($p < 0.05$). (B) Fresh and cryopreserved (frozen) wtNKG2D (wt) and chNKG2D (ch) T cells were cultured with myeloma cell lines for 24 hours, and cell-free CM were analyzed for IFN γ . Data are presented as mean + SD and are representative of 2 separate experiments. When cultured with tumor cells, fresh and cryopreserved chNKG2D T cells produced more IFN γ than wtNKG2D T cells ($p < 0.05$).