Chimeric NKG2D–Modified T Cells Inhibit Systemic T-Cell Lymphoma Growth in a Manner Involving Multiple Cytokines and Cytotoxic Pathways

Tong Zhang, Amorette Barber, and Charles L. Sentman

Department of Microbiology and Immunology, Dartmouth Medical School, Lebanon, New Hampshire

Abstract

In this study, the efficacy and mechanisms of chimeric NKG2D receptor (chNKG2D)–modified T cells in eliminating NKG2D ligand–positive RMA/Rae1 lymphoma cells were evaluated. Intravenous injection of RMA/Rae1 cells led to significant tumor formation in spleens and lymph nodes within 2 weeks. Adoptive transfer of chNKG2D-modified T cells after tumor injection significantly reduced tumor burdens in both spleens and lymph nodes, and prolonged the survival of tumor-bearing mice. Multiple treatments with chNKG2D T cells resulted in long-term tumor-free survival. Moreover, these long-term survivors were resistant to rechallenge with RMA tumor cells (NKG2D ligand–negative), and their spleen and lymph node cells produced IFN-γ in response to RMA but not to other tumors in vitro, indicating immunity against RMA tumor antigens. ChNKG2D T cell–derived IFN-γ and granulocyte-macrophage colony–stimulating factor, but not perforin (Pfp), tumor necrosis factor–related apoptosis-inducing ligand, or Fas ligand (Fasl) alone were critical for in vivo efficacy. T cells deficient in both Pfp and Fasl did not kill NKG2D ligand–positive RMA cells in vitro. Adoptive transfer of Pfp−/−Fasl−/− chNKG2D T cells had reduced in vivo efficacy, indicating that chNKG2D T cells used both mechanisms to attack RMA/Rae1 cells. Taken together, these results indicate that chNKG2D T-cell–mediated therapeutic effects are mediated by both cytokine-dependent and cytotoxic mechanisms in vivo. [Cancer Res 2007;67(22):11029–36]

Introduction

Immunotherapy has the potential to provide a highly selective means to control cancer in patients. Adoptive transfer of tumor antigen–specific T cells has shown promise in the treatment of cancers (1, 2). One of the major hurdles to successful T cell adoptive therapy is the difficulty in isolation and expansion of large numbers (>10⁹) of antigen-specific T cells (2, 3). To overcome this problem, alternative strategies to obtain a large number of tumor-specific T cells have been developed (4–8). A recent clinical trial showed that adoptive transfer of genetically engineered T cells with tumor-specific TCRs could lead to cancer regression in some patients (8). However, several factors, such as incorrect pairing between endogenous and exogenous TCR molecules, variable signaling capacity, MHC polymorphism, and down-regulation of MHC on target cells might limit the use of TCRs as immunotherapy (4–6). We previously described a strategy to genetically modify T cells with a chimeric natural killer (NK) receptor that comprises of a NK-activating receptor, NKG2D, linked to a gene encoding signaling domains of CD3ζ (9, 10). Murine or human T cells expressing such a chimeric receptor (chNKG2D) can recognize NKG2D ligand–positive tumor cells, become directly activated, secrete cytokines, and kill tumor cells.

A variety of effector mechanisms have been shown to be involved in immune responses against tumors. Direct cytotoxicity of tumors by NK cells or CTLs using perforin and/or FasL are important mechanisms for tumor elimination (11–13). In studies using spontaneous or transplanted tumors in perforin or FasL–deficient mice, there were higher incidences of tumors suggesting important roles for these host-derived molecules in the rejection of tumors. The requirement for donor T cell–derived perforin or Fasl for therapeutic efficacy in cell therapy models is still not well established. CTL-derived cytokines, especially Th1 cytokines, can also contribute to their antitumor effects by either stimulating the host immune system or directly inhibiting tumor cell growth (14–17). IFN-γ production by type I CD8+ T cells (Tc1) have been shown to be critical for Tc1-mediated therapeutic effects (18, 19). Thus, both cytotoxic effector pathways and cytokines may be involved in mediating protective effects against tumor growth.

In a subcutaneous RMA lymphoma model, we showed that adoptive transfer of chNKG2D-bearing T cells at the tumor site inhibited NKG2D ligand–bearing RMA (RMA/Rae1) tumor growth (9). Moreover, mice that had remained tumor-free were resistant to subsequent challenge with the wild-type RMA tumor cells, suggesting the generation of immunity against other tumor antigens (9, 20). In this study, we examined the therapeutic effects of chNKG2D T cells in a systemic lymphoma model using RMA/Rae1 cells given to mice i.v. The cytotoxic pathways and effector cytokine mechanisms responsible for therapeutic efficacy were also determined using T cells with specific deficiencies in perforin, Fasl, tumor necrosis factor–related apoptosis-inducing ligand (TRAIL), IFN-γ, or granulocyte-macrophage colony–stimulating factor (GM-CSF).

Materials and Methods

Mice. C57BL/6 (B6, wild-type, wt) mice were purchased from the National Cancer Institute. IFN-γ–deficient mice B6.129S7-Ifngtm1rs/J (IFN-γ−/−), Fas ligand–deficient mice B6.Smn.C3-Flsllgt/m Mouse Model (Fasl−/−), and perforin-deficient mice C57BL/6-Prf1tm1hsd/J (Pfp−/−) were obtained from the Jackson Laboratory, GM-CSF knockout mice (GM-CSF−/−) were obtained from Dr. Jeff Whitsett of the University of Cincinnati. TRAIL knockout mice (TRAIL−/−) were obtained from Dr. Jacques Peschon (Amgen, Seattle, WA). A mouse strain deficient for perforin and Fasl.
(Pp^-FasL^-) was generated by crossing Pp^- mice with FasL^- mice. The genotypes of the mice were determined by PCR of tail DNA. The primers for genotyping Pp^- mice are p1, 5'-GCTATCAGGACATAAGGTGCTTG-3' and p2, 5'-TACCAACAAATGGGCCCAAG-3'. The PCR products for Pp^- and Pp^- mice are 250 and 187 bp, respectively. The primers for Fasl mutation (FasL^¶) mice are f1, 5'-TGATCAATTTTGGGAACTAAGCC-3' and f2, 5'-ATAGTCTTAAGAGCTCATTCAAG-3'. The reactions for both PCRs were as follows: 95°C for 3 min, followed by 35 cycles of 95°C for 30 s, 56°C for 30 s, and 72°C for 30 s, ending with 72°C for 3 min. To identify the presence or absence of the Fasl mutation in FasL^-/- mice, 9 μL of the PCR product was digested with 10 units of Stul (NEB) for 3 h at 37°C. The resulting product was run on a 3% agarose gel, and the presence or absence of bands at 112 and 136 bp indicated the presence of a wild-type Fasl allele and a gld mutant Fasl allele, respectively. All experiments were conducted according to protocols approved by Dartmouth College's Institutional Animal Care and Use Committee.

**Tumor cell lines.** A RMA subline RMA/CG that coexpresses a NKG2D ligand, Rae1, and green fluorescent protein (GFP), was generated by retroviral transduction using dualtropic retroviral vectors containing the rae1/J and gfp genes according to our previous protocol (9). All cells were grown in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (HyClone), 100 units/mL of penicillin, 100 μg/mL of streptomycin, 1 mM of l-arginine, 10 mM of Hepes, 0.1 mM of nonessential amino acids, and 50 μM of 2-mercaptoethanol.

**Flow cytometry.** For fluorescence-activated cell sorting analysis of Rae1 expression, tumor cells were stained with APC-conjugated anti-Rae1 (R&D Systems). Cell surface phenotyping of transduced primary T cells was determined by staining with APC-conjugated anti-CD4 (clone 145-2C11; PharMingen), PE-conjugated anti-NKG2D (clone A10; eBioscience) and FITC-conjugated anti-CD8 (clone CT-CD8a; Invitrogen) monoclonal antibodies. To detect intracellular IFN-γ production by T cells, spleen cells (3 × 10^6) from naive B6 or tumor-surviving mice (125 days after tumor injection) were cultured with irradiated (120 Gy) RMA or ID8 cells (20:1) for 24 h. Intracellular IFN-γ staining was done as in our previous study using PE-conjugated anti-IFN-γ (clone XMG12; eBioscience) or PE-conjugated rat IgG1 isotype control (eBioscience; ref. 21). Surface markers of T cells were analyzed using FITC-conjugated anti-CD8 (clone CT-CD8a; eBioscience) and APC-conjugated anti-CD4 (clone RM4-5; BD Biosciences). All samples were preincubated with Fc receptor blocking antibody (antimouse CD16/CD32, clone 93; eBioscience) to reduce nonspecific staining. Cell fluorescence was monitored using a FACScan Calibur cytometer (Becton Dickinson).

**Cytotoxicity assays.** Cytotoxicity of chNKG2D-bearing T cells against target cells was determined by 5-h ³¹Cr release assay as previously described (9). To block perforin-mediated cytotoxicity, T cells were pretreated with concanamycin A (CMA, 20 mM; Sigma) for 2 h before an equal volume of target cells were added to cocultures.

**Treatment of mice with genetically modified T cells.** Retroviral transduction of murine primary T cells was done using ecotropic viruses, as we have previously described (9). C57BL/6 mice were injected with various numbers (2–5 × 10^6) of RMA/CG cells via tail veins in 400 μL of PBS. For treatment with T cells, mice were administered i.v. with 7.5 × 10^5 of wtNKG2D- or chNKG2D-modified T cells 2 days after tumor injection. Genetically modified T cells (6–7 days posttransduction) were harvested, washed, and resuspended in cold PBS before injection. Adoptively transferred T cells were a mixture of CD4^+ (70–90%) and CD8^+ (10–30%) T cells. For treatment with three doses of T cells, 5 × 10^6 T cells were given i.v. on days 2, 6, and 10 after tumor injection. For determination of tumor burdens in tumor-bearing mice, spleens and lymph nodes were collected 10 or 13 days after tumor injection. The lymphoid tissues were mechanically teased and washed. RBC were lysed with ACK lysis buffer (0.15 mol/L NH₄Cl, 1 mM mol/L KHCO₃, 0.1 mol/mL EDTA (pH 7.3)). The number of cells was counted, and the percentage of GFP^+ cells was determined by flow cytometry. The absolute number of tumor cells in these tissues was determined by multiplying the percentage of GFP^+ cells by the number of total cells. For survival studies, mice were monitored closely and sacrificed when moribund signs were observed.

**Tumor rechallenge.** Mice that survived for 75 days without signs of sickness were regarded as tumor-free and then were inoculated s.c. with 10^5 wild-type RMA cells on the shaved right flank. Naive B6 mice were used as controls. Tumor size was monitored every 2 days, and mice were sacrificed when tumor burden became excessive.

**IFN-γ production in secondary stimulation cultures.** Cultures of 10^6 lymph node or spleen cells from naive or tumor-surviving mice with 10^6 RMA or ID8 tumor cells were done in 48-well plates in 0.75 mL of complete medium. Tumor cells were irradiated (120 Gy) before use. Cell-free conditioned media were collected after 5 days and assayed for IFN-γ by ELISA using mouse DuoSet ELISA kits (R&D Systems) according to the instructions of the manufacturer.

**Statistical analysis.** Differences between groups were analyzed using Student's t test or ANOVA. P < 0.05 were considered significant. Kaplan-Meier survival curves were plotted and analyzed using Prism software (GraphPad Software).

**Results**

**Intravenous injection of RMA cells leads to systemic lymphoma.** Lymphoma is characterized by the infiltration of tumor cells in lymph nodes, spleens, and other lymphoid tissue. We...
used a systemic lymphoma model to mimic the clinical disease with widespread distribution of lymphoma cells into these lymphoid organs. To achieve this goal, we used a lymphoma cell line RMA/RG which expressed both a NKG2D ligand Rae1 and GFP, and injected the tumor cells i.v. into mice. Flow cytometry was done to determine the percentage of GFP+ tumor cells as well as the absolute numbers of tumor cells in various organs. As shown in Fig. 1A and B, 10 days after i.v. injection of 2 or 5 × 10^6 RMA/RG cells, GFP+ tumor cells could be readily detected in the spleen. Mice that were injected with 5 × 10^6 RMA/RG cells had larger tumor burdens than those which were injected with 2 × 10^6 tumor cells. The numbers of tumor cells increased rapidly thereafter. Thirteen days after injection of 5 × 10^6 RMA/RG cells, the percentages of tumor cells increased by >20-fold in spleens (from 1.2% to 27.8%) and 70-fold (from 0.025% to 1.95%) in lymph nodes, compared with that at day 10. A similar trend was observed in mice that were injected with 2 × 10^6 RMA/RG cells. Besides spleens and lymph nodes, RMA/RG cells also infiltrated bone marrow, thymus (Fig. 1C), and liver (data not shown).

ChNKG2D-modified T cells significantly reduce NKG2D ligand–positive tumor burden and promote long-term survival of mice in a systemic lymphoma model. To determine the efficacy of chNKG2D-bearing T cells in eliminating established tumors in a systemic lymphoma model, 2.5 × 10^6 RMA/RG cells were injected i.v. into B6 mice. Two days after tumor inoculation, 7.5 × 10^6 T cells (transduced with chNKG2D or wtNKG2D receptors) were administered i.v. As shown in Fig. 2, treatment with a single dose of chNKG2D-modified T cells significantly reduced both the percentages and absolute numbers of RMA/RG cells in the spleens and lymph nodes of tumor-bearing mice by 90% and 60%, respectively, compared with the treatment with wtNKG2D T cells. Besides reducing tumor burdens, the same treatment regimen using chNKG2D T cells also doubled the median survival from 15 to 30 days (Fig. 3A). Two of 16 (12.5%) mice became long-term survivors after one treatment with chNKG2D T cells. We observed even lower tumor burdens after three injections of 5 × 10^6 T cells (on days 2, 6, and 10; Fig. 2B and D). All mice that were treated with three doses of chNKG2D T cells remained alive >120 days after tumor injection (Fig. 3B). This result indicates that multiple treatments with fewer chNKG2D T cells was a better treatment regimen than a single dose. To determine whether a small tumor burden still existed in the lymphoid organs of long-term survivors, a nested-PCR analysis for a tumor-specific gene, GFP, was done on DNA samples extracted from the organs (Supplemental Fig. S1A). The sensitivity of this PCR is at least one tumor cell per reaction. All long-term survivor DNA samples from spleen, lymph node, or bone marrow were negative 125 days post–tumor inoculation for tumor DNA. The NKG2D ligand Rae1 expression on RMA/RG cells in vivo was also determined after treatment with either wtNKG2D or chNKG2D T cells. RMA/RG cells expressed lower levels of Rae1 in chNKG2D T cell–treated mice than in wtNKG2D-treated mice (Fig. 3C and D), suggesting that treatment with chNKG2D T cells might generate immune selection pressure and lead to survival of tumor “variants” that have down-regulated or lost Rae1 expression. However, multiple treatments with chNKG2D T cells resulted in long-term tumor-free survival.

Treatment with chNKG2D T cells induces the generation of memory responses against RMA cells that do not express ligands for NKG2D. Because NKG2D ligand–negative tumor cells may preferentially grow out, it would be beneficial if treatment with chNKG2D T cells induced host immunity against other tumor antigens. Eight of the mice that remained tumor-free after 75 days (Fig. 3B) were challenged s.c. with RMA tumor cells. These tumor-free mice were resistant to a subsequent challenge of wild-type RMA cells, whereas all control naïve mice had aggressive tumors (tumor area, ~120 mm^2) after 18 days (Fig. 4A). To test whether long-term tumor survivors generated specific memory responses against RMA cells, four tumor-free mice (Fig. 3B) were sacrificed 120 days post–tumor inoculation. Lymph node and spleen cells were cocultured with either RMA cells or ID8 tumor cells. ID8 is a B6-derived ovarian cancer tumor cell line (22). As shown in Fig. 4B–D, compared with the spleen and lymph node cells from naïve mice, tumor-free survivor-derived spleen and lymph node cells produced IFN-γ in response to stimulation by RMA but not...
cells. Both CD8+ and CD4+ T cells produced IFN-γ when cultured with RMA cells, but not with ID8 cells, as shown by intracellular staining (Fig. 4D). This memory T cell response was not mediated by host cells, not the adoptively transferred chNKG2D T cells, because there were no chNKG2D T cells present 120 days post–tumor inoculation based on a nested-PCR assay for the chimeric receptor gene (Supplemental Fig. S1B). These data indicate that adoptive transfer of chNKG2D T cells allowed hosts to generate immunologic memory against RMA tumor antigens.

Both perforin and Fas ligand, but not TRAIL, contribute to the cytotoxicity mediated by chNKG2D-bearing T cells in vitro. It is well known that perforin granule exocytosis and FasL-Fas interactions are two major mechanisms that CTLs use to kill target cells. RMA cells are Fas positive (23). To determine the mechanisms of cytotoxicity of RMA/Rae1 cells, we used T cells derived from Pfp−/−, Fasl−/− or TRAIL−/− mice as effector cells. There was a 5-fold reduction in the cytotoxicity against RMA/Rae1 cells by Pfp−/− or Fasl−/−-derived T cells (Fig. 5B). However, significant cytotoxicity remained in spite of the absence of perforin or Fasl. TRAIL deficiency did not affect the cytotoxicity of chNKG2D T cells. In addition, T cells that lacked IFN-γ or GM-CSF did not have reduced cytotoxicity against RMA/Rae1 cells (Supplemental Fig. S2). The killing of target cells by chNKG2D T cells is NKGD ligand–dependent because no specific killing of RMA cells was achieved by chNKG2D T cells (Fig. 5A and C). We have previously shown that blocking the NKGD receptor with anti-NKG2D antibodies prevented chNKG2D-mediated in vitro cytotoxicity (9, 10).

To pinpoint the relative contribution of each pathway to chNKG2D T cell cytotoxicity, we generated B6 mice deficient in both perforin and Fasl. Pfp−/− Fasl−/− chNKG2D T cells lack cytotoxic activity against RMA/Rae1 cells (Fig. 5D), indicating that the combination of perforin and Fasl accounted for all of the chNKG2D-bearing T cell–mediated cytotoxicity against RMA/Rae1 cells. This result was further confirmed by combining CMA, which is an inhibitor of the perforin-dependent pathway (24), with the use of Fasl−/− T cells. Similar to the observation obtained using perforin-deficient T cells, the addition of CMA alone led to a reduction but not total abrogation of cytotoxicity of tumor cells (Supplemental Fig. S3). However, CMA-treated Fasl-deficient chNKG2D T cells had no cytotoxic activity against RMA/Rae1 cells (Supplemental Fig. S3). These results indicate that chNKG2D-bearing T cells use both FasL and perforin to kill RMA/Rae1 tumor cells.

Direct killing of RMA/Rae1 cells by chNKG2D T cells is involved in their in vivo efficacy. Considering the important roles of perforin and Fasl in chNKG2D T cell–mediated in vitro cytotoxicity, we tested whether these two effector molecules were also involved in chNKG2D T cell–mediated therapeutic efficacy in vivo. ChNKG2D-bearing T cells derived from either Pfp−/−, Fasl−/−, or B6 mice were adoptively transferred into RMA/RG-bearing B6 mice. ChNKG2D T cells derived from either Pfp−/− or Fasl−/− mice displayed an equivalent capacity as T cells from B6 mice to reduce tumor burden (Fig. 6A), indicating that either perforin or Fasl alone was not required for in vivo therapeutic effects. TRAIL-deficient chNKG2D T cells did not show any reduced functionality in therapeutic effects in vivo, which was in line with the fact that TRAIL did not contribute to the in vitro cytotoxic activity. As shown in Supplemental Fig. S4A and C, Pfp−/− or Pfp−/−/Fasl−/− chNKG2D T cells produced similar amounts of IFN-γ in response to RMA/Rae1 cells as B6 chNKG2D T cells. Thus, deficiency in these cytotoxic pathways did not inhibit other effector functions. However, T cells deficient in both perforin and Fasl showed reduced therapeutic efficacy in vivo. The data indicate that chNKG2D T cell–mediated direct cytotoxicity of tumor cells is involved in the in vivo effects, although either perforin or Fasl alone are not required for chNKG2D T cells to be effective.

Donor cell–derived IFN-γ and GM-CSF are critical for chNKG2D T-cell–mediated therapeutic effects. Cytokines may activate host immune cells and alter the local tumor microenvironment. To determine whether cytokines from chNKG2D T cells were involved in their in vivo antitumor effects, we used T cells derived from mice with defects in Ifn-γ or gm-csf. We have previously shown that chNKG2D T cells produce IFN-γ and

![Image](Cancer Res 2007; 67: (22). November 15, 2007 11032 www.aacrjournals.org)
Adoptive transfer of chNKG2D T cells induces memory responses. A. tumor-free mice (C) in the chNKG2D-treated group (shown in Fig. 3B) and naive mice (A) were challenged with wild-type RMA cells (10^6) s.c. into the right flank. Points, mean of tumor areas; bars, SE (P < 0.05 on days 6–18). Lymph node (B) and spleen cells (C) from tumor survivors (Fig. 3B) produced significantly higher (P < 0.05) IFN-γ than cells from naive mice after stimulation with irradiated RMA cells (filled columns). Only background amounts of IFN-γ production were observed in lymph nodes or spleen cells when cultured with ID8 cells (hatched columns) or media alone (open columns). D, Intracellular production of IFN-γ by CD8^+ or CD4^+ T cells upon culture with RMA (filled columns), ID8 (hatched columns), or media only (open columns) for 24 h (*, P < 0.05).

**Discussion**

T cells have a highly restrictive antigen specificity through their TCR, which limits their antitumor activity to cells that express the particular antigen. In addition, enough MHC expression of the proper allele on tumor cells is required to ensure significant killing by T cells. Therefore, down-regulation of MHC on tumor cells helps tumor cells evade T cell recognition (25). NKG2D ligands are primarily expressed on tumor cells but not normal cells (26). Thus, the chNKG2D receptor–NKG2D ligand system provides a relatively specific system for T cells to recognize tumor cells and become fully activated irrespective of tumor cell MHC/tumor antigen expression. Many human leukemia and lymphoma cells have been found to express NKG2D ligands (27). It has been shown that the antileukemia effect of NK cells in leukemia involves NKG2D-target cell interactions (28). Therefore, the generation of systemic lymphomas by i.v. administration of RMA/Rae1 tumor cells is a good model to mimic human lymphomas.

GM-CSF upon culture with tumor cells that express NKG2D ligands (9, 10). Although IFN-γ is not directly linked to chNKG2D T cell–mediated cytotoxicity as shown in *in vitro* cytotoxic assays (Supplemental Fig. S2A), *in vivo* it may activate immune cells, such as macrophages, dendritic cells, and/or lymphocytes to achieve therapeutic effects. As shown in Fig. 6B, administration of IFN-γ-deficient chNKG2D T cells failed to reduce tumor burden, indicating the critical role for IFN-γ production by these transferred cells *in vivo*. GM-CSF-deficient chNKG2D T cells had an impaired ability to reduce tumor burdens compared with chNKG2D T cells, although these T cell–treated mice had lower tumor burdens than the mice treated with IFN-γ–deficient chNKG2D T cells. This impaired *in vivo* activity was not due to a lack of IFN-γ production because GM-CSF deficiency did not affect the T cell production of IFN-γ (Supplemental Fig. S4B). In addition, a lack of GM-CSF did not affect T cell cytotoxicity (Supplemental Fig. S2B). Thus, the production of cytokines was critical for the antitumor responses mediated by chNKG2D T cells *in vivo*.

Although NK cells are able to recognize tumors using a variety of activating receptors, their *in vivo* activities are dampened by many different inhibitory receptors (29). Our preliminary data showed that chNKG2D-bearing T cells lack the expression of inhibitory receptors such as Ly49A or Ly49C/I.1 In addition, chNKG2D T cells predominantly produce Th1 cytokines, not Th2 cytokines, whereas NK cells can express Th2 cytokines such as interleukin (IL)-10 and transforming growth factor-β, which may inhibit their antitumor activity (30, 31). Therefore, chNKG2D-bearing T cells are expected to achieve more significant antitumor effects than NK cells.

ChNKG2D T cells show cytotoxicity against RMA/Rae1 cells *in vitro*. This cytotoxic activity uses both perforin and FasL pathways. Expression of either one of these two molecules conferred killing of RMA/Rae1 cells. Similarly, neither perforin nor FasL alone was required for chNKG2D T cell–mediated *in vitro* antitumor activity. It is interesting that even though deficiencies in these cytotoxic pathways caused a 5-fold drop in cytotoxicity *in vitro*, they had no effect on the efficacy of chNKG2D T cells *in vivo*. A study by Winter et al. showed similar results in which effector T cell–mediated tumor regression following adoptive transfer was independent of perforin or FasL (32). The reasons for these results may be that both perforin- and FasL-mediated pathways could be used for cytotoxicity. Using chNKG2D T cells generated from Pfp^−/−/Fasl^−/− mice, we showed that direct killing was involved in the chNKG2D T cell–mediated antitumor effects *in vivo*. Unlike the critical role of IFN-γ generated by chNKG2D T cells in controlling tumor burden, chNKG2D T cells that could not kill were still able to reduce tumor burden by >70%. Thus, cytotoxicity by chNKG2D T cells is important, but chNKG2D T cells promote tumor elimination even in the absence of their cytotoxic function.

Although chNKG2D T cells produce multiple cytokines and chemokines, including IL-3, CCL3, and CCL5 *in vitro* upon engagement with RMA/Rae1 cells (9), IFN-γ production was essential to...
mediate antitumor effects by chNK2D T cells in vivo. It is well-known that IFN-γ coordinates a diverse array of cellular programs (14, 33). In some models, IFN-γ has been shown to be critical in inhibiting tumor growth by inducing apoptosis or inhibiting angiogenesis (34, 35). Corthay et al. showed that IFN-γ was critical for CD4+ T cell–mediated macrophage activation and tumor rejection (36). Cytokines (such as IL-12, IL-18, and IL-21) and costimulatory molecules (CD40 and LIGHT) have also been shown to mediate antitumor effects in an IFN-γ–dependent manner (34, 37–39). In this systemic lymphoma model, we speculate that the IFN-γ production by chNK2D T cells upon recognition of NK2D ligand–positive tumor cells may activate host immune cells (macrophages, dendritic, NK, and/or T cells) and enhance the development of Th1 responses leading to systemic antitumor effects in vivo. Although not as critical as IFN-γ, GM-CSF was necessary for maximum therapeutic benefit because GM-CSF−/− chNK2D T cells did not promote tumor clearance as well T cells that produced GM-CSF. GVAX, a GM-CSF gene-transduced tumor vaccine, has been shown to be immunostimulatory and to have antitumor activity in cancer patients in several clinical trials (40). GM-CSF may recruit and activate dendritic cells and macrophages, thereby improving tumor antigen presentation. In this model, direct killing of RMA/Rae-1 tumor cells by chNK2D-bearing T cells in situ may help release tumor antigens in combination with concurrent production of GM-CSF, which may provide a good milieu for the recruitment of dendritic cells and antigen presentation.

This study, and our previous results, showed that adoptive therapy of RMA/Rae1 tumor-bearing mice with chNK2D T cells could elicit hosts to generate memory responses against NK2D ligand–negative RMA cells and these responses required host adaptive immunity (9, 20). Generation and maintenance of memory responses are important for reducing recurrent tumor disease because (a) memory T cells respond to recall antigens at a much faster pace and greater magnitude than naïve T cells; and (b) memory T cells survive much longer than their naïve counterparts (41, 42). Tumor cells are also known to mutate (15); therefore, induction of polyclonal tumor–specific T cells will minimize the chances of tumor cells to “escape.” Generation of memory T cells can be negatively affected by an immunosuppressive environment in tumor-bearing hosts (43–45). Regulatory T cells and immunosuppressive cytokines such as transforming growth factor-β and IL-10 have been shown to impair memory generation as well as effector functions (43–45). In contrast, proinflammatory cytokines and Toll-like receptor agonists are able to rescue antitumor memory responses in immunosuppressive environments (45, 46). Therefore, it is possible that chNK2D T cell–tumor cell interaction may create a proinflammatory environment, thus helping overcome immunosuppressive conditions and promoting the generation of memory T cells.

One of the hurdles to adoptive T cell transfer for cancer immunotherapy is that most infused cells failed to persist to mediate an effective response (47). An easy means to enhance the activity and survival of transferred cells is administration of cytokines, such as IL-2, IL-7 and IL-15, etc (48–50). These cytokines share the common γ chain receptor and regulate lymphocyte growth and survival. The administration of low, nontoxic doses of IL-2 and IL-7 can improve the survival of transferred cells and lead to the establishment of persistent T cell memory (48, 49). Although we observed significant therapeutic

![Figure 5](image-url)
effects with chNKG2D T cell–infusion without coadministration of cytokines, we expect that cytokine administration in combination with chNKG2D-bearing T cell transfer may give better results in tumor inhibition.

Antigen-specific T cells represent heterogeneous populations of differentiated cells that include effector, effector-memory, and central-memory subsets that differ in functional properties and the differential expression of homing receptors and costimulatory molecules (51). Recent data showed that administration of naive and early effector T cells, in combination with active immunization and IL-2, resulted in the eradication of large, established tumors (52). However, more-differentiated effector T cells were less effective for in vivo tumor treatment. In our study, genetically modified T cells were transferred into mice within 10 days after initial activation to avoid potential reduced functional activity. Phenotypically, these chNKG2D T cells are early effectors (i.e., CD62L+/−, FasL+/−, TRAIL+/−, or Pip+/− FasL+/− mice) at day 2. B, tumor-bearing mice that were inoculated i.v. with 2.5 × 10⁶ RMA/RG cells on day 0 were treated with a single dose of 7.5 × 10⁶ chNKG2D T cells derived from either wild-type B6, IFN-γ−/−, or GM-CSF−/− mice on day 2. wtNKG2D T cells from B6 mice were used as negative controls. The percentages of GFP+ cells in spleens were determined by flow cytometry on day 13. Points, data pooled from two independent experiments. Significance was determined by ANOVA. **, P < 0.01, compared with B6 wtNKG2D T cell treatment; †, P < 0.01, compared with B6 chNKG2D T cell treatment.

**References**


Acknowledgments

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Unpublished data.

Figure 6. The effector mechanisms of chNKG2D T cell–mediated therapeutic effects in vivo. A, tumor-bearing mice that were inoculated i.v. with 2.5 × 10⁶ RMA/RG cells on day 0 were treated with a single dose of chNKG2D T cells (7.5 × 10⁶) derived from either wild-type B6, Pip−/−, TRAIL−/−, or Pip−/− FasL−/− mice on day 2. wtNKG2D T cells from B6 mice were used as negative controls. The percentages of GFP+ cells in spleens were determined by flow cytometry on day 13. Points, data pooled from two independent experiments. Significance was determined by ANOVA. **, P < 0.01, compared with B6 wtNKG2D T cell treatment; †, P < 0.01, compared with B6 chNKG2D T cell treatment.

B6

wtNKG2D

chNKG2D

TRAIL−/−

Fasl−/−

Pip−/−

B6

chNKG2D

chNKG2D

chNKG2D

chNKG2D

chNKG2D