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Immunotherapy with Chimeric NKG2D Receptors Leads to Long-Term Tumor-Free Survival and Development of Host Antitumor Immunity in Murine Ovarian Cancer¹,²

Amorette Barber, Tong Zhang, and Charles L. Sentman³

Ovarian cancer is one of the leading causes of cancer death in women and the development of novel therapies is needed to complement the standard treatment options such as chemotherapy and radiation. In this study, we show that treatment with T cells expressing a chimeric NKG2D receptor (chNKG2D) was able to lead to long-term, tumor-free survival in mice bearing established ovarian tumors. Tumor-free mice were able to reject a rechallenge with ovarian tumor cells 225 days after original tumor injection. In addition, chNKG2D T cell treatment induced specific host immune responses to ovarian tumor cells, including the development of both CD8⁺ and CD4⁺ T cell tumor-specific memory responses. The chNKG2D T cells reduced the ovarian tumor burden using both cytotoxic and cytokine-dependent pathways. Specifically, chNKG2D T cell expression of perforin, GM-CSF, and IFN-γ were essential for complete antitumor efficacy. The Journal of Immunology, 2008, 180: 72–78.

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Materials and Methods

Mice

Female C57BL/6 (B6) mice were purchased from the National Cancer Institute (Frederick, MD). IFN-γ-deficient B6.129S7-Ifng<sup>−/−</sup> (IFN-γ<sup>−/−</sup>), FasL-deficient B6Smn.C5-Fasl<sup>−/−</sup> (FasL<sup>−/−</sup>), and perforin-deficient C57BL/6-Prf1<sup>−/−</sup> mice were purchased from The Jackson Laboratory.

Abbreviations used in this paper: chNKG2D, chimeric NKG2D; wtNKG2D, wild-type NKG2D.

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Laboratory. GM-CSF knockout mice (GM-CSF-/-) on a C57BL/6 background were obtained from Dr. J. Whitsett (University of Cincinnati, Cincinnati, OH). Mice used were between 6 and 8 wk of age. All animal work was performed in the Dartmouth Medical School Animal Facility in accordance with institutional guidelines.

**Injection of ID8-GFP cells and treatment of mice with genetically modified T cells**

Mouse spleen cells were isolated, stimulated with Con A for 18 h (1 μg/ml), and transduced as previously described (14). Two days after transduction, T cells were selected in medium containing G418 (0.5 mg/ml) and 25 U/ml recombinant human IL-2 for 3 days. Viable cells were isolated using hepatoma-1085 (Sigma-Aldrich) and expanded for 2 days without G418 before functional analyses, as previously described (14). wtNKG2D and chNKG2D T cells consist of both CD8+ (90%) and CD4+ (10%) T cells. wtNKG2D and chNKG2D T cells were generated from B6 or immunodeficient mouse spleen cells, and similar cell yields and expressions of NKG2D were obtained from the different strains. ID8-GFP cells were cultured as previously described (12). For survival studies, female B6 mice were injected with 2 × 10^6 ID8-GFP cells i.p. in 500 μl of PBS, wtNKG2D or chNKG2D modified T cells (5 × 10^5) were injected into mice i.p. 1, 2, and 3 wk after tumor injection. Mice were weighed at least once a week and observed until they had gained ~80% of their original body weight. For chNKG2D T cell mechanism studies, female B6 mice were injected with 2 × 10^6 ID8-GFP cells i.p. and treated 1 wk later with 5 × 10^5 wtNKG2D or chNKG2D modified T cells generated from B6, IFN-γ−/−, GM-CSF−/−, or perforin−/− mice. Eight weeks after receiving the tumor injection, mice were sacrificed and a peritoneal wash was performed using 10 ml of PBS. The number of solid tumors on the peritoneum was counted. RCC in the peritoneal washes was lysed with ACK lysis buffer (0.15M NH4Cl, 1 mM KHCO3, 0.1 mM 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 the peritoneal washes was determined by multiplying the percentage of GFP+ cells by the number of cells in the peritoneal wash. For the ID8-GFP tumor challenge, 10^6 ID8-GFP cells were injected i.p. into naive B6 or tumor-surviving mice. After 10 wk, mice were sacrificed and the absolute number of GFP+ cells in the peritoneal wash and number of solid tumors on the peritoneum were determined.

**DNA isolation and nested PCR**

Total DNA was isolated from spleen cells and peritoneal wash cells using a lysis buffer (50 mM Tris·HCl, 100 mM NaCl, 0.1% Triton X-100, and 1 mg/ml protease K) at 5 × 10^6 cells/30 μl. Cells were lysed at 55°C overnight, and proteinase K was inactivated at 98°C for 10 min. These samples were directly used as template for nested PCR. The amplification of the tumor-specific gfp gene, primers were 5'PB (5'-ATCCTCTAGA CTGCAGATCG-3') and 3'PB (5'-CATATTGGAAAAAGCAGCAT ATG-3'), which are specific for the retroviral vector pFB-neo (Stratagene). Cycling conditions consisted of a 3-min denaturation at 94°C, followed by 30 cycles of amplification (each cycle consisting of a 30-s denaturation at 94°C, a 30-s annealing at 56°C, and a 45-s extension at 72°C) and a final 3-min extension at 72°C. Nested amplifications used 1 μl of the primary PCR product as the template. The nested PCR primers were 5'GP (5'-CA CATTGAAGCAGCAGACTT-3') and 3'GP (5'-GGCTCAGGTAGTGG TTGTCG-3'), which are specific for the gfp gene. Nested cycling conditions were the same as described above, except for a 45-s annealing at 59°C, and a 45-s extension at 72°C. For amplification of the chNKG2D gene, the pFB-neo primers described above were used to amplify the retroviral vector. Amplification conditions were similar to the pFB-neo conditions described above, only with a 45-s annealing at 59°C, and a 45-s extension at 72°C. Nested amplifications used 500 μl of each of DNTP, 1 U of Taq polymerase (NEB), and 0.2 μM primers. The PCR products were run on agarose gels and visualized by staining with SYBR Safe (Invitrogen Life Technologies).

**Intracellular cytokine detection by flow cytometry**

Spleen cells (2.5 × 10^5) from naive or tumor-surviving mice were cultured with irradiated ID8 or RMA cells (2.5 × 10^5) for 72 h. Cell-free conditioned medium was assayed for IFN-γ by ELISA using mouse Duoset ELISA kits (R&D Systems).

**Cytokine production in secondary stimulation cultures**

Intracellular cytokine detection by flow cytometry

Irradiated ID8 or RMA cells (1.5 × 10^5) were cultured with spleen cells (3 × 10^5) from naive B6 or tumor-surviving mice for 24 h. During the last 5 h of culture, 10 μg/ml brefeldin A was added to the wells (Sigma-Aldrich). Cells were then incubated with FcR block and cell surface stained with FITC-conjugated anti-CD8 (clone CT-CD8; eBioscience) and allopurinol-conjugated anti-CD4 (clone RM4-5, BD Biosciences) and permeabilized with permeabilization buffer containing FcR block, paraformaldehyde, monensin, and brefeldin A (eBioscience), PE-conjugated anti-CD4 (clone RM4-5, BD Biosciences) and allopurinol-conjugated anti-CD8 (clone CT-CD8; eBioscience) and stained with PE-conjugated anti-IFN-γ (clone XMG12; eBioscience) and allopurinol-conjugated anti-TNF-α (clone MP6-XT22; BioSource International), or PE-conjugated anti-rat IgG1 isotype control (eBioscience). Cell fluorescence was monitored using a FACSCalibur cytometer (BD Biosciences).

**Cytotoxicity by chNKG2D T cells**

Lysis of ID8 cells was determined by ^51Cr-release assays as previously described (14, 15).
Statistical analysis

Differences between groups were analyzed using the Student t test or ANOVA. Values of p < 0.05 were considered significant. For survival studies, Kaplan-Meier survival curves were plotted and analyzed using Prism software (GraphPad Software).

Results

Adoptive transfer of chNKG2D T cells to ID8 tumor-bearing mice leads to long-term tumor-free survival

The murine ovarian cell line, ID8, was originally derived from spontaneously transformed B6 mouse ovarian surface epithelial cells and upon injection the tumor cells form ascites and solid tumors on the peritoneum in an immunocompetent host that mimics stage III/IV ovarian cancer (16). ID8 tumor cells express the NKG2D ligand Rae1 on the cell surface, but they do not express H-60 or Mult-1 (12). To determine whether treatment with chNKG2D T cells prolonged survival of mice with an established ID8 tumor burden, 2 or 5 × 10⁶ ID8-GFP tumor cells were injected i.p. into mice, and the mice were given chNKG2D- or wtNKG2D-bearing T cells after 1, 2, and 3 wk. The survival of the mice was determined and mice were sacrificed when they had gained 80% of their original body weight due to ascites formation (Fig. 1, A and B). At both tumor doses, all of the chNKG2D T cell treated mice survived to 225 days, when a majority of the mice were removed from the experiment for analysis of immune function. Four mice at each tumor dose were allowed to continue until 325 days (Fig. 1A). At 325 days, all surviving mice were healthy. The median survival of the wtNKG2D T cell-treated mice was 97 days for those given 5 × 10⁶ tumor cells and 120 days for mice given 2 × 10⁶ tumor cells. Although wtNKG2D T cell-treated mice gained a significant amount of weight, chNKG2D T cell-treated mice did not have any unexpected weight gain or measurable ascites formation (Fig. 1B).

To address whether there was a low level of tumor burden remaining after chNKG2D T cell treatment, a nested PCR assay for a tumor-specific gene, gfp, was performed on peritoneal washes of FIGURE 2. chNKG2D T cell-treated mice develop host immune responses to ID8 cells. A, PCR for the chNKG2D gene was performed on the DNA from spleen cells of chNKG2D T cell-treated surviving mice 230 days after original tumor challenge or naive B6 mice. Contents in each lane are from: lanes 2–4, three surviving mice from 2 × 10⁶ ID8-GFP challenge; lanes 5–7, three surviving mice from 5 × 10⁶ ID8-GFP challenge; lanes 8–10, three naive mice; lane 11, water; lane 12, 100 wtNKG2D T cells; lane 13, 100 chNKG2D T cells; lane 14, 10 chNKG2D T cells; lane 15, 1 chNKG2D T cell; lanes 1 and 16, 100-bp marker. B, A total of 230 days after tumor injection, spleen cells from three surviving mice from each group from Fig. 1A and three naive B6 mice were cultured with medium alone (□), ID8 cells (■), or RMA cells (□). Cell-free supernatants were analyzed for IFN-γ. Significant differences are indicated (*, p < 0.001). Data are represented as mean ± SD.

FIGURE 3. Tumor-surviving mice develop host T cell memory responses to ID8 Ags. Spleen cells from chNKG2D T cell-treated mice surviving 325 days after tumor injection (n = 4 from each group from Fig. 1A) and naive B6 mice (n = 3) were cultured with medium (□), ID8 (■), or RMA (□). After 24 h, intracellular cytokine staining was performed for (A) IFN-γ and (B) TNF-α production and gated on CD8+ or CD4+ T cells as indicated. T cells from tumor surviving mice produced significantly more IFN-γ and TNF-α when cultured with ID8 cells than naive mice, as indicated (*, p < 0.05).
three surviving mice 230 days after tumor injection. This PCR assay was able to detect at least one ID8-GFP cell equivalent in the PCR sample (Fig. 1C). No amplification of gfp was detected in the peritoneal wash cells or spleen cells of these mice (data not shown); therefore, chNKG2D T cell-treated surviving mice were ID8-GFP tumor cell-free 230 days after tumor injection. Thus, treatment with chNKG2D T cells led to long-term, tumor-free survival in a murine model of ovarian cancer.

**Treatment with chNKG2D T cells induces a protective host antitumor immune response against ID8 tumor cells**

It has been shown in other tumor models that survival of adoptively transferred T cells is correlated with antitumor in vivo efficacy (1, 17, 18). To determine whether chNKG2D T cells are being maintained long-term in vivo, a nested PCR assay specific for chNKG2D was performed. The spleens of chNKG2D T cell-treated surviving mice from Fig. 1A did not contain any chNKG2D T cells 230 days after tumor injection (Fig. 2A). This indicated that the chNKG2D T cells were not maintained long-term in vivo, although the mice remained long-term survivors. To determine whether chNKG2D T cell-treated surviving mice have developed host immune responses to ID8 cells, spleen cells from surviving mice or from naive B6 mice were cultured with ID8 cells, RMA cells (a B6-derived lymphoma), or with medium alone. Spleen cells from surviving mice secreted IFN-γ when cultured with ID8 cells, but not with RMA cells or medium (Fig. 2B).

Naive spleen cells did not secrete IFN-γ when cultured in any of the conditions. These data indicate the development of a host immune response to ID8 Ags in tumor-surviving mice.

To characterize the development of a host T cell memory response to tumor Ags, intracellular cytokine analysis was performed on spleen cells from naive B6 or surviving mice 325 days...
after tumor injection (from Fig. 1A). IFN-γ and TNF-α production was analyzed in spleen cells cultured with ID8 cells, RMA cells, or medium. A significant population of both CD8⁺ and CD4⁺ cells from surviving mice secreted IFN-γ and TNF-α after coculture with ID8 cells, but not RMA cells or medium alone (Fig. 3). The percentage of CD8⁺ T cells secreting these cytokines was higher than the CD4⁺ T cell population. T cells from naive mice did not produce these cytokines. The percentage of CD8⁺ and CD4⁺ cells staining positive for TNF-α was high; however, we cannot rule out the possibility that some of the positive cells were due to cell surface binding of TNF-α.

To test whether these memory responses were protective against a rechallenge with ID8 tumor cells, naive B6 and surviving mice (from each group in Fig. 1A) were challenged with 10⁶ ID8-GFP cells i.p. After 10 wk, tumor burden was evaluated. Although naive B6 mice had a large number of tumor cells in the peritoneal wash and solid tumors on the peritoneum, 9 of 10 tumor-surviving mice completely rejected the rechallenge with tumor cells, and one mouse had a very low tumor burden (Fig. 4). Taken together, these data show that treatment with chNKG2D T cells led to the establishment of host antitumor immunity, with generation of both CD8⁺ and CD4⁺ ID8-specific T cells and protection against an ID8 tumor challenge.

**ChNKG2D T cells require both cytotoxicity and cytokines for in vivo efficacy**

We have shown that chNKG2D T cells can both secrete cytokines and lyse ovarian tumor cells in vitro; however, the molecular mechanisms chNKG2D T cells require to decrease ovarian tumor burden in vivo are unknown (12). Previous studies using anti-NKG2D-blocking Abs have shown that chNKG2D T cell lysis of tumor cells is dependent on NKG2D recognition (12, 14, 15). CD8⁺ T cells are able to use multiple cytotoxic pathways to directly kill tumor cells, including perforin and Fas-FasL. Which pathway effectively kills the tumor cell often depends on tumor cell susceptibility or resistance to these killing pathways. To first determine the in vitro mechanisms that chNKG2D T cells use to kill ID8 cells, chNKG2D T cells were derived from B6 mice, perforin⁻/⁻, or FasL⁻/⁻ mice, and these cells were used as effectors against ⁵¹Cr-labeled ID8 cells (Fig. 5A). FasL⁻/⁻ chNKG2D T cells were able to lyse ID8 cells as well as chNKG2D T cells from B6 mice. However, chNKG2D T cells deficient in perforin were unable to kill ID8 cells, indicating that chNKG2D T cells use perforin but not FasL, to kill ID8 cells.

Although chNKG2D T cells require expression of perforin to kill ID8 cells in vitro, the effector mechanisms required to eliminate tumor burden in vivo likely require both cytotoxicity and cytokine production. To test the in vivo effector mechanisms required for chNKG2D T cell antitumor activity, chNKG2D T cells were derived from B6-, perforin-, IFN-γ-, or GM-CSF-deficient mice, and used to treat ID8-GFP tumor-bearing mice (Fig. 5, B and C). chNKG2D T cells deficient in perforin were able to significantly reduce the tumor burden compared with wtNKG2D T cell-treated mice, but not near as well as chNKG2D T cell-treated mice. Strikingly, chNKG2D T cells deficient in IFN-γ were unable to reduce the tumor burden, illustrating the requirement of chNKG2D T cell-derived IFN-γ for ovarian tumor reduction. In addition, chNKG2D T cell-derived GM-CSF also plays a role in tumor reduction, with GM-CSF-deficient T cells able to reduce the tumor burden partially, but not completely. The loss of efficacy by the knockout-derived chNKG2D T cells was not due to a deficiency in other effector functions, as the IFN-γ⁻/⁻ and GM-CSF⁻/⁻ chNKG2D T cells were able to lyse ID8 cells as well as chNKG2D T cells from a B6 mouse. Perforin⁻/⁻ and GM-CSF⁻/⁻ chNKG2D T cells secreted similar amounts of IFN-γ as B6-derived chNKG2D T cells when cultured with tumor cells (Fig. 6). Taken together, chNKG2D T cells require both cytotoxic function and cytokine secretion to eliminate an established ID8 tumor.

**Discussion**

We have shown that treatment of an ovarian tumor with chNKG2D T cells leads to tumor-free survival. Additionally, the surviving mice developed a host memory response to ID8 tumor cells, and this host response was able to protect against a rechallenge with the same ovarian tumor cells. Complete tumor elimination by chNKG2D T cells required T cell-derived IFN-γ, GM-CSF, and perforin, indicating that both cytotoxicity and cytokine secretion play a role in the in vivo response.

Currently, there is much research on using adoptive transfer of tumor Ag-specific T cells as a potential therapy for cancer. Initial trials involving transfer of tumor-infiltrating lymphocytes after nonmyeloablative chemotherapy in melanoma patients showed
significant efficacy (19–21). However, many other recent clinical trials, including one in ovarian cancer using patient T cells genetically modified with a chimeric receptor specific for the α-folate receptor, were not as successful (2). Many clinical trials use T cells that are at a late effector phase due to long-term activation and expansion in vitro before transfer to the patients. However, it has recently been shown that treatment with fewer T cells that are at an earlier effector phase are more effective at decreasing tumor burden (1, 22, 23). The chNKG2D T cells are at an early effector phase based on cell surface staining (our unpublished data) and so may have stronger antitumor ability. Furthermore, many chimeric receptors engineered from single-chain Ab fragments can be immunogenic and thus rejected or inhibited by the host immune response (2, 24, 25). However, the extracellular portion of the chNKG2D receptor consists of the wtNKG2D receptor and so should not result in an immune response against the receptor.

Although chNKG2D T cells did not persist long-term in the tumor-surviving mice, this treatment did lead to the induction of host T cell memory responses to tumor Ags. The memory response that developed in these mice was protective against a tumor rechallenge, as mice that were injected with ID8 tumor cells 225 days after the original tumor challenge were able to reject the tumor rechallenge, illustrating the development of a functional memory response to ovarian tumor Ags. One indication that the development of host memory responses was specific is that both CD8+ and CD4+ T cells specifically produced IFN-γ and TNF-α when cultured with ID8 cells, but not with B6-derived T cell lymphoma cells or medium alone. The development of host memory responses to tumor Ags may be important for tumor immunity, as immune responses to additional Ags would be beneficial if the tumor cells down-regulate NKG2D ligand expression or if not all of the tumor cells express NKG2D ligands. The percentages of cells that stained positive for TNF-α were surprisingly high, but it is possible that TNF-α that was secreted in the cell cultures bound to the cell surface receptors for TNF and represented both cell-bound and production of TNF-α.

Although T cells are able to kill tumor cells through multiple mechanisms, we showed that chNKG2D T cells depend solely on perforin to kill ID8 cells in vitro as chNKG2D T cells deficient in perforin expression were not able to lyse ID8 cells. Despite inability to kill in vitro, perforin−/− chNKG2D cells were still able to significantly, but not completely, reduce the ovarian tumor burden in vivo. However, chNKG2D T cell-derived IFN-γ was essential for reducing the tumor burden as T cells deficient in this cytokine could not reduce the tumor burden. Although the exact mechanisms of chNKG2D T cells need to be further investigated, it has been shown in other tumor systems that IFN-γ is able to have many antitumor effects such as activating macrophages, inhibiting angiogenesis, increasing MHC class I expression, promoting Ag presentation, and having antiproliferative effects on tumor cells (26–30). Multiple clinical trials have shown that administering IFN-γ in combination with chemotherapy can be an effective treatment for ovarian cancer patients (31–33). In addition, chNKG2D T cell-derived GM-CSF, a molecule involved in the recruitment and activation of APCs, also had a role in tumor reduction. Ongoing clinical trials in ovarian cancer include administering GM-CSF to patients, and some trials that have been completed showed efficacy when GM-CSF was provided alone or in combination with other treatments such as chemotherapy (34, 35). Thus, both direct cytotoxicity and cytokine secretion by chNKG2D T cells are involved in complete antitumor efficacy. It is possible that chNKG2D T cells not only directly kill tumor cells, thereby decreasing the tumor burden and releasing tumor Ags, but also activate and recruit APCs, such as dendritic cells and macrophages, helping to initiate host immune response to additional tumor Ags, and lead to the development of a host immune response that is involved in eliminating tumor cells and results in the development of T cell memory responses. In addition, the myriad of suppressor cells and molecules found in the tumor environment, such asIDO, programmed death-1 ligand-1, myeloid suppressor cells, and regulatory T cells, often suppress T cell responses that develop in ovarian cancer patients (36–39). The proinflammatory environment that chNKG2D T cells create around the tumor may also be acting to relieve these immunosuppressive mechanisms.

Ovarian cancer is difficult to treat and has the highest mortality rate of all the cancers of the female reproductive tract. This is partially due to the lack of early symptoms and frequent diagnosis at a late stage of disease. Over 50% of cases are found when a patient is at least in stage III of disease, when the cancer has metastasized to the peritoneal lining and/or the lymph nodes. The ID8 tumor model used in this study recapitulates many aspects of ovarian cancer including the formation of both ascites and solid tumors on the peritoneum. Treatment of an established ID8 tumor with chNKG2D T cells led to tumor-free survival and the development of functional host T cell memory responses to ovarian tumor Ags.

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Disclosures
The authors have no financial conflict of interest.

References


Corrections


We recently discovered through single-nucleotide polymorphism analysis that the GM-CSF–deficient mice used in these studies were not backcrossed onto the C57BL/6 (B6) background and appear to be a B6/129/FVB genetic mix. These mice have the MHC region of B6 origin. Because it is theoretically possible that the other genes in this mixed strain could account for some of the phenotypes we observed using these mice, we have done several experiments to test this hypothesis. We have performed in vivo experiments on acute chimeric Ag receptor (CAR) T cell effects and anti-tumor efficacy studies using 129/Sv, (B6 × 129/Sv)F1, or C3H as sources of CAR T cells, and these F1 and allogeneic CAR T cells gave similar readouts as B6 CAR T cells in the same experiments with B6 recipients. GM-CSF–deficient mice as hosts in the ID8 ovarian tumor model or the RMA-RG lymphoma model resulted in a similar outcome as when B6 CAR T cells are used in B6 hosts. These data suggest that different background genes do not affect the outcomes in these types of experiments. However, we cannot rule out that a unique combination of genes in this strain may have some effect, so we want the scientific community to be aware that the GM-CSF–deficient mouse strain used in these was of a mixed genetic background.

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