Chimeric NKG2D T Cells Require Both T Cell- and Host-Derived Cytokine Secretion and Perforin Expression to Increase Tumor Antigen Presentation and Systemic Immunity

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Chimeric NKG2D T Cells Require Both T Cell- and Host-Derived Cytokine Secretion and Perforin Expression to Increase Tumor Antigen Presentation and Systemic Immunity1,2

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Treatment of mice bearing established ovarian tumors with T cells expressing chimeric NKG2D receptors (chNKG2D) develop protective host immune responses to tumor Ags. In this study, the mechanisms that chNKG2D T cells require to induce host immunity against ovarian tumors and which of the host immune cells are involved in tumor elimination were determined. Treatment with chNKG2D T cells led to a sustained, increased IFN-γ production by host NK, CD4+ T, and CD8+ T cells in the spleen and at the tumor site and this continued for many weeks after T cell injection. Tumor Ag presentation was enhanced in chNKG2D T cell-treated mice, and there were greater numbers of tumor-specific T cells at the tumor site and in draining lymph nodes after treatment with chNKG2D T cells. The increase in host cell cytokine secretion and Ag presentation was dependent on chNKG2D T cell-derived perforin, IFN-γ, and GM-CSF. Host immune mechanisms were involved in tumor elimination because inhibition of tumor growth was limited in mice that lacked perforin, IFN-γ, NK cells, or T and B cells (Rag1−/−). There was no role for host-derived GM-CSF or CD1-dependent NKT cells, because mice deficient in these were able to clear tumors as well as treated wild-type B6 mice. In summary, chNKG2D T cells required both cytotoxicity and cytokine secretion as well as the participation of host immune cells for development of a host antitumor immune response and complete efficacy. The Journal of Immunology, 2009, 183: 2365–2372.

Adoptive T cell transfer has the potential to provide effective specific therapy for cancer. It has been found that prior leukodepletion of patients and long-term survival of the transferred T cells may enhance antitumor efficacy (1, 2). However, Ag escape variants and down-regulation of MHC class I can ultimately lead to evasion of immune response and eventual tumor progression in patients (3). Development of a host immune response that targets a variety of tumor Ags may help prevent the outgrowth of Ag-loss tumor variants and improve clinical outcome.

One benefit of using T cell therapies for treatment of cancer is that T cells can specifically target tumor cells with limited cross-reaction with normal tissues. T cells recognize tumor Ags as peptides associated with MHC molecules, or T cells can be engineered to recognize Ags directly through chimeric receptors. Many current immunotherapy trials target a variety of tumor-associated Ags including melanoma Ags, cancer testes Ags, p53, Her-2/neu, and α-folate receptor through use of T cells expressing specific receptors for tumor Ags (3, 4). Another potential tumor-specific target are the ligands for the NKG2D receptor. NKG2D is an activating receptor expressed on NK cells, CD8+ T cells, and subsets of other T cells (5). The ligands for this receptor are expressed on many different types of carcinomas and hematopoetic tumors and are not expressed on the cell surface of most normal tissues (6, 7).

Induction of host cell production of IFN-γ at the ovarian tumor site may be one way to increase antitumor immunity. Treating ovarian cancer patients with a combination of IFN-γ and chemotherapy has shown some benefits and IFN-γ has many antitumor mechanisms, including activating APCs, increasing Ag presentation by tumor cells, and having cytostatic effects on tumor cells directly (8–10). In addition, increasing the activation of host APCs to induce host T cell and NK cell responses to tumor Ags may also improve cancer therapy (11).

Previously, we reported on a chimeric NKG2D receptor which consists of the NKG2D receptor fused to the cytoplasmic region of the CD3ζ chain (12–16). Treatment of mice bearing established ovarian cancer with chNKG2D T cells led to long-term, tumor-free survival (16). These tumor-surviving mice had developed host CD4 and CD8 cell memory responses to tumor Ags, and this immune response was protective against tumor rechallenge (16). This indicates that chNKG2D T cell therapy induces the development of host immune responses to tumor Ags. In this study, we determined the mechanisms whereby chimeric NKG2D (chNKG2D)4 T cells activate host immune responses to tumor Ags, examining host cell IFN-γ production, Ag presentation, and trafficking of tumor-specific T cells. We also determined the requirement of host molecules in the development of the host immune response and tumor elimination.

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

Mice

Female C57BL/6 (B6) and B6-LY5.2/Cr (CD45.1<sup>+</sup>) mice were purchased from the National Cancer Institute. B6.Rag1<sup>−/−</sup> mice were bred and maintained at Dartmouth Medical School. C57BL/6 IFN-γ-deficient B6.129S7-Ifng<sup>−/−</sup> mice, IFN-γ receptor 1-deficient B6.129S7-IfngR1<sup>−/−</sup> mice, perforin-deficient C57BL/6-Prf1<sup>−/−</sup> mice, and C57BL/6-Tg[cx-tcln<sup>100MIP</sup>]OT-1<sup>−/−</sup> mice were purchased from The Jackson Laboratory. GM-CSF-deficient mice (GM-CSF<sup>−/−</sup>) on a C57BL/6 background were provided by Dr. J. Whitsett (University of Cincinnati) and B6.CD1<sup>−/−</sup> mice were provided by Dr. M. Exley (Harvard University, Boston, MA). Mice were used between 7 and 12 wk of age at the start of the experiments. 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 cultured with Con A for 18 h (1 μg/ml) and transduced as previously described (13, 14). Two days after transduction, T cells were selected in medium containing G418 (0.5 mg/ml) and 25 μ/ml recombinant human IL-2 for 3 days. Viable cells were isolated using Histopaque-1083 (Sigma-Aldrich) and expanded for 2 days without G418 (13, 14). ID8-GFP cells (2 × 10<sup>6</sup>) were injected i.p. into B6 or mice deficient in IFN-γ, GM-CSF, perforin, Rag1, or CD1. Mice were treated i.p. with wild-type NKG2D (wtNKG2D) or chNKG2D T cells (5 × 10<sup>6</sup>) 1 wk after tumor injection. Mice were sacrificed and a peritoneal wash was performed using 10 ml of PBS. The number of solid tumors on the peritoneal wall was counted. RBC in the peritoneal washes were lysed with ACK lysis buffer (0.15 M NH<sub>4</sub>Cl, 1 mM KHCO<sub>3</sub>, and 0.1 mM EDTA, pH 7.3), the number of cells was counted, and the percent GFP<sup>+</sup> cells was determined by flow cytometry. Absolute number of tumor cells in the peritoneal washes was determined by multiplying percent GFP<sup>+</sup> cells by the number of cells in the peritoneal wash.

In vivo NK cell depletion

To deplete NK cells, 200 μg of anti-NK1.1 (PK136) or control mouse γ-globulin (Jackson Immunoresearch Laboratories) was injected into mice i.p. 2 days before and 3 days after T cell injection (day +5 and day +10 relative to tumor cell injection).

Cytokine secretion and intracellular cytokine detection by flow cytometry

Peritoneal wash cells (10<sup>6</sup>) from tumor-bearing wtNKG2D or chNKG2D T cell treated mice were cultured in 48-well plates in complete medium. Twenty-four hour cell-free conditioned medium was assayed for IFN-γ by ELISA using mouse DuoSet ELISA kits (R&D Systems) and for NO using Griess’s reagent for nitrite (Sigma-Aldrich) on a C57BL/6 background. For intracellular cytokine detection, frozen spleens or mediastinal lymph node cells were isolated. CD8<sup>+</sup> OT-1 T cells were purified from spleen and lymph node cells using magnetic bead selection (Milenyi Biotec) and FITC-conjugated anti-CD8<sup>+</sup> Abs according to the manufacturer’s instructions, and purity was >95% for OT-1 T cells (CD8<sup>+</sup>). CFSE-labeled OT-I T cells (5 × 10<sup>5</sup>) were cultured with spleen and lymph node cells (10<sup>3</sup>) and proliferation of T cells was determined by flow cytometry after 72 h. For peptide pulsing, spleen and lymph node cells were incubated with OVA<sub>257-264</sub> peptide (10<sup>−9</sup>–10<sup>−11</sup> M) for 2 h at 37°C, and the cells were washed three times to remove unbound peptides before culture with OT-1 T cells.

In vivo OT-I T cell trafficking and survival

ID8-GFP-Ova or ID8-GFP cells (2 × 10<sup>6</sup>) were injected i.p. into B6 mice, and after 7 days mice were treated i.p. with 5 × 10<sup>6</sup> wtNKG2D or chNKG2D T cells. At the same time as T cell injection, purified, CFSE-labeled OT-I T cells (2 × 10<sup>5</sup>) that were congenically marked with both Ly5.1 and Ly5.2 were injected i.v. Four days after transfer of OT-I T cells, a peritoneal wash was performed, spleen cells and mediastinal lymph node cells were processed, and the presence of Ly5.1<sup>−</sup>Ly5.2<sup>+</sup> CFSE-labeled OT-I T cells was determined by flow cytometry.

Statistical analysis

Differences between groups were analyzed using the Student t test or ANOVA using Prism software (Graphpad Software). Values of p < 0.05 were considered significant.

Results

Treatment of tumor-bearing mice with chNKG2D T cells induces cytokine secretion from host immune cells

Treatment of mice bearing an established ovarian tumor burden with chNKG2D T cells led to long-term, tumor-free survival and the induction of host memory responses to tumor Ags (16). However, the mechanism of how chNKG2D T cells induce systemic immune responses against tumor Ags is unclear. To study the development of the host immune response, ID8-GFP ovarian tumor cells were injected i.p. into mice and were treated 1 wk later with T cells expressing chNKG2D receptors or wtNKG2D receptors i.p. One, 3, and 7 days after T cell injection, spleen cells from treated mice were cultured with medium, and the supernatants were analyzed for IFN-γ production (Fig. 1). Spleen cells from mice treated with chNKG2D T cells secreted significantly more IFN-γ compared with mice treated with wtNKG2D T cells. The increase in IFN-γ secretion from spleen cells began 3 days after T cell treatment, with a peak at 7 days after chNKG2D T cell injection. Similar data were found when mice were treated with chNKG2D T cells 5 wk after tumor inoculation (data not shown). To determine which cell types secreted IFN-γ, intracellular cytokine staining was performed (Fig. 1B and supplemental Fig. 1), chNKG2D T cells were not a source of IFN-γ since these T cells did not traffic to the spleen after i.p. injection, as shown by the lack of Ly5.1<sup>+</sup> cells in the spleen at any time point analyzed (Fig. 1B). There was an increase in the production of IFN-γ by host NK cells, CD8<sup>+</sup> and CD4<sup>+</sup> T cells 3 and 7 days after chNKG2D T cell injection, indicating that treatment with chNKG2D T cells led to an increased number of host cells that secreted IFN-γ.

In mice with an established tumor burden, a single treatment with chNKG2D T cells led to a significantly reduced tumor burden compared with mice treated with wtNKG2D T cells (14). The kinetics of the host IFN-γ response were analyzed to determine whether the induced host immune response was sustained for long periods of time. The increased IFN-γ secretion in chNKG2D T cell-treated mice was maintained up to 10 wk after T cell injection, and both lymphocytes at the tumor site and in the spleen were producing IFN-γ (Fig. 2A). This response decreased over time with the peak IFN-γ response occurring 7 days after T cell injection.

<sup>5</sup>The online version of this article contains supplemental material.
Host cells from the peritoneal cavity also secreted a significant amount of NO for at least 4 wk after chNKG2D T cell injection. Spleen cells did not secrete NO at any time point analyzed (data not shown). Similar to earlier time points, host NK, CD8⁺/H11001, and CD4⁺/H11001 T cells from chNKG2D T cell-treated mice produced more IFN-γ compared with mice treated with wtNKG2D T cells, and this response decreased over time.

Because the secretion of additional cytokines may be affected by chNKG2D T cell treatment, cytokine production by peritoneal and spleen cells from wtNKG2D or chNKG2D T cell-treated mice was analyzed (Table I). Seven weeks after chNKG2D T cell injection, cells isolated from the tumor site and the spleen secreted elevated amounts of many cytokines, including IL-2, GM-CSF, CCL3, and CCL4. Additionally, there was a decrease in KC and CCL2 in mice treated with chNKG2D T cells. Together, these data demonstrate that treatment of tumor-bearing mice with chNKG2D T cells induced a sustained host immune response and proinflammatory cytokine production from NK and T cells at the tumor site and in the spleen.

**Induction of IFN-γ production from host cells requires chNKG2D T cell-derived cytokines and perforin**

It has been shown that chNKG2D T cell-derived IFN-γ, GM-CSF, and perforin are essential for complete antitumor efficacy.
The requirement for cytokine secretion and tumor lysis may be partially due to their involvement in inducing a systemic host immune response to tumor Ags. Tumor-bearing mice were treated with chNKG2D T cells derived from wild-type B6 mice or from mice deficient in IFN-γ, GM-CSF, or perforin. chNKG2D T cell-derived IFN-γ, GM-CSF, and perforin were all required for a complete induction of an IFN-γ response, as shown by decreased IFN-γ secretion from spleen cells both 3 and 7 days after treatment with chNKG2D T cells deficient in any of these effector molecules (Fig. 3A). Since chNKG2D T cell-derived IFN-γ could be acting directly on the tumor cells or on the host immune cells, mice deficient in the IFN-γR were used as hosts to determine whether host cell responsiveness to IFN-γ was required. An increase in IFN-γ secretion was not observed after chNKG2D T cell treatment in mice deficient in the IFN-γR, indicating that host cells needed to respond to the IFN-γ secreted by the chNKG2D T cells for the induction of the host immune response (Fig. 3B).

Intracellular staining was performed on spleen cells to determine which host cells were affected by chNKG2D T cell-derived molecules. chNKG2D T cell-derived GM-CSF was required for the induction of host NK cell and CD4+ T cell IFN-γ production, while chNKG2D T cell-derived IFN-γ and perforin were required for the induction of host CD8+ and CD4+ T cell responses, as shown by a decreased percentage of IFN-γ+ cells from mice treated with T cells deficient in these molecules (Fig. 3C). Thus, chNKG2D T cell-derived cytokines and perforin were involved in the induction of a systemic host antitumor response.

Treatment with chNKG2D T cells increased tumor Ag presentation and tumor-specific T cell infiltration at the tumor site

Treatment with chNKG2D T cells may increase host immune responses to ID8 tumor cells by increasing Ag presentation and therefore the activation of host T cells. To test this, mice were injected with ID8-GFP cells that expressed a truncated OVA gene (ID8-GFP-Ova) that is not secreted and therefore remains an intracellular tumor Ag (18, 19). After 7 days of tumor growth, mice were treated with either PBS, wtNKG2D, or chNKG2D T cells. One week after T cell injection, spleen cells and draining lymph node cells were isolated and cultured with CFSE-labeled OT-I T cells, which recognize the tumor-specific OVA peptide presented by MHC class I molecules. After 3 days of culture, OT-I T cells proliferated more when cultured with spleen or lymph node cells from chNKG2D T cell-treated mice compared with mice treated with wtNKG2D T cells or with PBS (Fig. 4A). Spleen and lymph node cells from wtNKG2D- and chNKG2D T cell-treated mice had similar numbers of APCs, including CD11c+ dendritic cells and macrophages, and these APCs expressed similar amounts of MHC class I, MHC class II, CD80, and CD86 (data not shown). This suggests that spleen and lymph node cells from the chNKG2D T cell-treated mice were presenting more Ag to the OT-I T cells. To determine whether the increase in OT-I T cell proliferation was dependent on specific Ags, similar experiments were performed with mice bearing ID8-GFP tumors that did not express OVA. OT-I T cells proliferated more when cultured with spleen and lymph node cells from chNKG2D T cell-treated mice than with cells from wtNKG2D- or PBS-treated mice, but the increased proliferation was modest compared with OT-I T cells that were cultured with cells from chNKG2D T cell-treated mice bearing tumors that expressed OVA (Fig. 4). In addition, lymph node and spleen cells from chNKG2D T cell-, wtNKG2D T cell-, and PBS-treated mice were pulsed with increasing concentrations of OVA peptides which induced proliferation of OT-I T cells (Fig. 4C and data not shown). This indicates that chNKG2D T cells induced T cell proliferation because of increased Ag presentation and other additional factors, likely cytokine secretion, from spleen and lymph node cells. The increase in tumor Ag presentation was dependent on chNKG2D T cell-derived GM-CSF, IFN-γ, and perforin as cells isolated from mice treated with chNKG2D T cells deficient in one of these molecules did not have increased Ag presentation by cells taken from the draining lymph node or spleen (Fig. 4D).

To address how chNKG2D T cell treatment affects tumor Ag-specific T cells in vivo, wtNKG2D or chNKG2D T cells were injected i.p. into ID8-GFP-Ova bearing mice and concurrently mice were injected with CFSE-labeled OT-I T cells i.v. Three days after OT-I T cell transfer, the trafficking of the tumor Ag-specific T cells was measured. A higher number of OT-I T cells was found in the draining lymph node and at the tumor site in the chNKG2D
T cell-treated mice compared with mice treated with wtNKG2D T cells or PBS, demonstrating that chNKG2D T cells also increased in vivo trafficking and/or survival of tumor Ag-specific T cells at the tumor site and in draining lymph nodes (Fig. 5). To determine whether this T cell recruitment was Ag specific, trafficking of OT-I T cells was measured in mice bearing ID8-GFP tumors that did not express OVA. Compared with mice treated with wtNKG2D T cells or PBS, treatment with chNKG2D T cells did not increase trafficking of OT-I T cells to the tumor-draining lymph node, indicating that the increase in OT-I T cells at the lymph node was Ag dependent. Although an increased number of OT-I T cells was found in the peritoneal cavity in mice that received chNKG2D T cells, this increase was not as great as that seen in mice bearing tumors expressing OVA. This suggests that chNKG2D T cells increased T cell trafficking to the tumor site in both Ag-dependent and Ag-independent manners, possibly through an alteration in chemokine production, as shown in Table I.

ChNKG2D T cells require host lymphocytes and IFN-γ and perforin from host cells for in vivo efficacy

Although chNKG2D T cells induced a systemic host immune response to tumor Ags, the requirements of host components for tumor elimination were unknown. To investigate the role of the host immune system in this response, wild-type B6 mice or mice deficient in perforin, GM-CSF, or IFN-γ were injected with ID8 tumor cells and treated 1 wk later with wtNKG2D or chNKG2D T cells. Wild-type B6 mice treated with chNKG2D T cells had a significantly reduced number of tumor cells in the peritoneal cavity and solid tumors compared with mice treated with wtNKG2D T cells after 8 wk (Fig. 6). Mice deficient in IFN-γ had no reduction in tumor burden after chNKG2D T cell treatment, indicating that host cell-derived IFN-γ was essential for chNKG2D T cell antitumor efficacy. Mice deficient in perforin had a reduced tumor burden after chNKG2D T cell treatment, but not as low as recipient mice that expressed perforin, indicating that perforin expression by
host cells was also required for complete antitumor efficacy. Although chNKG2D T cell-derived GM-CSF was required for reducing the tumor burden, chNKG2D T cells were able to completely reduce the tumor burden in mice that could not produce GM-CSF, demonstrating that the host did not require expression of GM-CSF for tumor elimination.

Many cell types in the mouse express perforin and IFN-γ, including T cells and NK cells. To examine the requirement of different host lymphocytes, tumor-bearing Rag1-deficient mice (lacking T cells and B cells) were treated with wtNKG2D or chNKG2D T cells. ChNKG2D T cells significantly reduced the tumor burden in Rag1−/− mice, but not as effectively as in mice with intact T and B cells, indicating a role for both chNKG2D T cells and host lymphocytes in antitumor efficacy (Fig. 7A). Rag1−/− mice treated with wtNKG2D T cells had lower tumor burden than B6 mice, which may reflect the enhanced NK cell activity present in these mice (20). A similar effect was observed if NK cells were depleted, showing that host NK cells were involved in decreasing the tumor burden after chNKG2D T cell injection (Fig. 7B). Host CD1-dependent NKT cells were not required for antitumor efficacy because chNKG2D T cells were able to reduce the tumor burden in CD1−/− hosts to the same extent as in wild-type B6 mice (Fig. 7C). Overall, chNKG2D T cell reduction of an established tumor burden required components of the host immune system for best efficacy, including host-derived perforin and host lymphocytes.

Discussion
Transfer of chNKG2D T cells to mice bearing ovarian cancer led to the induction of a host immune response against the tumor. This included an increase in host NK cell and CD8+ and CD4+ T cell responses, and these responses were sustained for up to 10 wk after chNKG2D T cell injection. chNKG2D T cell treatment also increased Ag presentation in tumor-draining lymph nodes and in the spleen and increased tumor-specific T cell homing to the lymph nodes and tumor site in vivo. The induction of the host immune response and Ag presentation by chNKG2D T cells required their production of IFN-γ, GM-CSF, and perforin. Complete antitumor efficacy also required host cell-derived IFN-γ and perforin and host lymphocytes.

Currently, there are many T cell therapy approaches being developed for cancer treatment. In some of these studies, it was found that a nonmyeloablative chemotherapy regimen and radiation treatment before transfer of tumor-specific T cells enhanced antitumor efficacy (1, 21, 22). A possible reason for this is that depleting a patient of immune cells before T cell transfer decreases competition for APCs and homeostatic cytokines such as IL-15, which may allow for the transferred T cells to survive long term (23–25). Also, these pretreatment regimens may affect regulatory cells, such as myeloid-derived suppressor cells and regulatory T cells which may allow for the transferred T cells to survive long term. Although many of the current T cell therapies aim to induce the survival of their transferred T cells, this study shows that although chNKG2D T cells do not live long term after injection, they instead induce long-lived antitumor immune responses in the host. This induction of a host immune response...
may be beneficial for many reasons. If the tumor cells decrease the expression of NKG2D ligands or if there were some tumor cells present in a patient that did not express NKG2D ligands, then the tumor cells may be able to escape direct recognition by the transferred T cells. However, the induction of a host immune response to tumor Ags may result in epitope spreading and the development of immunity against multiple tumor Ags. Thus, chNKG2D T cells may induce a response to ligand-negative tumor cells through induction of host antitumor immune responses. Furthermore, the fact that the chNKG2D T cells do not survive long term may be also good for safety reasons. Some patients that have tumor Ag-specific T cells that survive long term show signs of autoimmunity due to Ag expression on normal tissues (27). Although the ligands for NKG2D are not expressed on most normal tissues, cell surface expression can be up-regulated in some situations, such as after infection with certain microbes (28, 29). This could be potentially dangerous if chNKG2D T cells survived long term as these T cells may then recognize and attack normal tissues if NKG2D ligand expression was expressed at the cell surface.

Components of the endogenous immune system may also be involved in the reduction of ovarian tumor growth. In Fig. 1, intracellular staining showed that although chNKG2D T cells increased the NK cell secretion of IFN-γ, NK cells in wtNKG2D T cell-treated mice also produced IFN-γ, indicating that the host responded to the tumor, but this response was not sufficient for tumor elimination. chNKG2D T cells increased host IFN-γ production 3 days after T cell injection, which suggests that the chNKG2D T cell response may enhance an ongoing host response. In addition, wtNKG2D T cell-treated mice that were deficient in IFN-γ or NK cell depleted had a somewhat increased tumor burden compared with B6 mice. This indicates that these host mechanisms may play a role in controlling tumor growth; however, these clearly cannot control tumor growth by themselves. chNKG2D T cells likely act together with host immune cells to overcome local immune suppression and lead to tumor elimination.

Although the role of host immune components in T cell adoptive therapies is not well understood, the data presented here demonstrate that host cells play an important role in antitumor efficacy of the transferred T cells. chNKG2D T cell treatment induced the sustained secretion of many proinflammatory cytokines even 10 wk after chNKG2D T cell injection. Although three doses of chNKG2D T cells leads to tumor-free survival, one dose of chNKG2D T cells may not completely eliminate the tumor (14, 16). Therefore, host immune cells may be continually responding to a low level of residual tumor present after a single chNKG2D T cell treatment, as tested here. Without host cell-derived perforin or lymphocytes, the antitumor efficacy of chNKG2D T cells was decreased. Thus, the chNKG2D T cells did not act alone to reduce the tumor burden, but instead they elicited the host immune system to respond against the tumor. Consequently, chNKG2D T cell therapy provides both passive immunity and also has a tumor vaccine-like effect in supporting the induction of active host antitumor immunity.

chNKG2D T cells increased tumor Ag presentation in both the draining lymph node and spleen of tumor-bearing mice. The increase in Ag presentation depended not only on chNKG2D T cell-derived perforin, which may be acting to lyse tumor cells, thus releasing more Ag for APCs, but also chNKG2D T cell-derived GM-CSF and IFN-γ. These cytokines may mature dendritic cells at the tumor site and cause them to traffic to the draining lymph node to initiate host T cell responses (30). An increased number of tumor Ag-specific T cells were found in the draining lymph node and at the tumor site in chNKG2D T cell-treated mice compared with mice treated with wtNKG2D T cells or PBS. This could be due to a combination of increased proliferation, survival, or trafficking of tumor Ag-specific T cells in chNKG2D T cell-treated mice.

Understanding the mechanisms that transferred T cells use to decrease tumor burden increases our understanding of how to improve on T cell immunotherapies for cancer. This study shows that transfer of chNKG2D T cells was able to initiate the induction of a host immune response in a mouse model of ovarian cancer. The longevity of the immune response to tumor Ags did not require the survival of chNKG2D T cells, but instead induced a proinflammatory environment through cytokine secretion and increased tumor Ag presentation to induce an effective host-dependent antitumor response.

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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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Supplementary Figure 1. Host NK cells and T cells have increased IFNγ production after treatment with chNKG2D T cells. Seven days after T cell injection, spleen cells from wtNKG2D (WT) or chNKG2D T cell (CH) treated tumor bearing mice were cultured in media for 24 hours and cells were assayed for IFNγ production by intracellular staining. Cells were gated on either (A) CD8+CD3+ or (B) NK1.1+CD3- and were stained for IFNγ or isotype control. Plots are representative of one mouse of each group (n=4). Data are representative of at least 5 separate experiments.