Chimeric NKG2D Expressing T Cells Eliminate Immunosuppression and Activate Immunity within the Ovarian Tumor Microenvironment

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Adoptive transfer of T cells has shown therapeutic potential in some types of cancer, such as melanoma and EBV-derived tumors (1–3). However, the response rate of T cell immunotherapy is relatively low in other types of cancer, including ovarian cancer (4). One contributing factor for the poor response to T cell immunotherapy may be due to the types of immune cells present at the tumor site. Leukocytes that may have antitumor activities can be found at the tumor site, including CD8⁺ T cells and NK cells (5). However, ovarian cancer also has many types of immunosuppressive cells in the tumor microenvironment, including myeloid-derived suppressor cells, vascular leukocytes, and regulatory T (Treg) cells (5–7). Elevated levels of immunosuppressive molecules such as PD-L1, IDO, PGE₂, IL-10, and arginase may also inhibit the immune response against tumors (8–10). Therapies that alter leukocyte populations in the tumor microenvironment to prevent the function of immunosuppressive populations and induce the recruitment and activation of immune cells may lead to the development of long-lived antitumor immune responses and improve cancer therapy.

Therapeutic efforts to induce immune responses in ovarian cancer include administering inflammatory cytokines, such as IFN-γ or GM-CSF alone or in combination with chemotherapy, and these treatment strategies have shown some therapeutic success (11–14). Additionally, treatments that inhibit immunosuppressive populations, such as inhibiting Treg cells using denileukin diftitox which consists of IL-2 fused to diptheria toxin, or using CTLA-4 blocking Abs, have increased antitumor immune responses in ovarian cancer patients (15, 16).

Transfer of tumor-reactive T cells has the potential to both kill tumor cells and activate the immune response through proinflammatory cytokine secretion. It has been shown that adoptive transfer of T cells expressing chimeric NKG2D (chNKG2D) receptors, which consist of the NKG2D receptor fused to the cytoplasmic domain of TCR-γ or GM-CSF alone or in combination with chemotherapy, and these treatment strategies have shown some therapeutic success (11–14). Additionally, treatments that inhibit immunosuppressive populations, such as inhibiting Treg cells using denileukin diftitox which consists of IL-2 fused to diptheria toxin, or using CTLA-4 blocking Abs, have increased antitumor immune responses in ovarian cancer patients (15, 16).

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Adoptive transfer of T cells expressing chimeric NKG2D (chNKG2D) receptors, a fusion of NKG2D and CD3ζ, can lead to long-term, tumor-free survival in a murine model of ovarian cancer. To determine the mechanisms of chNKG2D T cell antitumor efficacy, we analyzed how chNKG2D T cells altered the tumor microenvironment, including the tumor-infiltrating leukocyte populations. chNKG2D T cell treatment of mice bearing ID8 tumor cells increased the number and activation of NK cells and increased the activation of host CD8⁺ T cells within the tumor. Foxp3⁺ regulatory T cells at the tumor site decreased more than 300-fold after chNKG2D T cell treatment. Tumor-associated regulatory T cells expressed cell surface NKG2D ligands and were killed by chNKG2D T cells in a perforin-dependent manner. chNKG2D T cells also altered the function of myeloid cells at the tumor site, changing these cells from being immunosuppressive to enhancing T cell responses. Cells isolated from the tumor produced elevated amounts of IFN-γ, NO, and other proinflammatory cytokines after chNKG2D T cell treatment. ChNKG2D T cells required perforin, IFN-γ, and GM-CSF to induce a full response at the tumor site. In addition, transfer of chNKG2D T cells into mice bearing tumors that were established for 5 weeks led to long-term survival of the mice. Thus, chNKG2D T cells altered the ovarian tumor microenvironment to eliminate immunosuppressive cells and induce infiltration and activation of antitumor immune cells and production of inflammatory cytokines. This induction of an immune response likely contributes to chNKG2D T cells’ ability to eliminate established tumors. The Journal of Immunology, 2009, 183: 6939–6947.
Materials and Methods

Mice
Female C57BL/6 (B6) mice and B6-LY5.2/Cr (CD45.1) mice were purchased from the Jackson Laboratory. Mice deficient for both perforin and FasL were generated as previously described (18). GM-CSF-deficient mice on a C57BL/6 background were provided by Dr. Jeff Whitsett (University of Cincinnati, Cincinnati, OH). Mice used were 7 to 10 weeks of age at the start of each experiment. All animal work was performed in the Dartmouth Medical School Animal Facility (Lebanon, NH) in accordance with Institutional guidelines.

Injection of ID8-GFP cells and treatment of mice with genetically modified T cells
Mouse spleen cells were stimulated with Con A for 18 h (1 μg/ml) and transduced as previously described (19, 20). 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 Histopaque-1083 (Sigma-Aldrich) and expanded for 2 days without G418 (19, 20). At the time of transfer, wild-type NKG2D (wtNKG2D)- and chNKG2D-transduced splenocytes were 99% CD3+/NK1.1−, were a mixture of CD4+ (80–90%) and CD8+ (10–20%) cells, and had increased expression of NKG2D (21). ID8-GFP cells (2 × 10^6) were injected i.p. into B6 or IFN-γ−/− mice. wtNKG2D or chNKG2D T cells (5 × 10^6) were transferred i.p. at 1 or 5 weeks after tumor injection. Mice were killed and peritoneal washes were performed using 10 ml PBS. RBC in the peritoneal washes were lysed with ACK lysis buffer and the number of cells was counted. For survival experiments, ID8-GFP cells (2 × 10^6) were injected i.p. into B6 mice and wtNKG2D or chNKG2D T cells (5 × 10^6) were transferred i.p. 5, 6, and 7 weeks or 5, 7, and 9 weeks after tumor injection. Mice were weighed at least once a week and were killed if they had gained more than 80% of their original body weight.

Detection of tumor infiltrating populations by flow cytometry
Cells isolated by peritoneal wash were incubated with anti-CD16/CD32 and mouse γ globulin (Jackson Immunoresearch Laboratories), and stained with FITC-conjugated anti-CD3 (clone 145–2C11), anti-CD11c (clone N418), or anti-CD4 (clone GK1.5); PE-conjugated anti-CD3, anti-IFN-γ, CD8 (clone 53–6.7), anti-CD4 (clone 14–25), or anti-CD19 (clone ID3); allophycocyanin-conjugated anti-CD45.1 (clone A20, clone BM8), or anti-NK1.1 (clone PK136); and biotin-conjugated anti-CD69, anti-CD45.1 (clone A20) and biotin-conjugated anti-NK1.1 (clone M5/114,15,2). Anti-CD8 (clone eBio500A2), with a PE-Cy5.5 conjugated-streptavidin secondary. Cells were fixed with 1% paraformaldehyde, permeabilized with 0.1% saponin, and stained with PE-conjugated anti-Foxp3 (clone MF-14, BioLegend) or isotype control Abs. All Abs were purchased from eBioScience unless otherwise noted. Cell fluorescence was monitored using a FACScalibur cytometer (BD Biosciences).

To test for in vitro killing of CD4+Foxp3+ and CD19+ cells, peritoneal wash cells were isolated from mice bearing ID8-GFP cells for 8–9 weeks. Peritoneal wash cells (2 × 10^6) were cultured with 1 × 10^6 wtNKG2D or chNKG2D T cells generated from a B6 mouse, or chNKG2D T cells generated from mice deficient in perforin, Fasl, or both perforin and Fasl. After 24 h, the percent CD4+Foxp3+ cells and CD19+ cells was evaluated by flow cytometry.

T cell proliferation assay
Three days after T cell transfer, tumor-bearing mice were killed and a peritoneal wash was performed. Peritoneal wash cells from naive mice were used as a control. F4/80+ cells were isolated from peritoneal washes using biotin-conjugated anti-F4/80 Abs and magnetic bead selection (Miltenyi Biotec) according to the manufacturer’s instructions. CD8+ OT-I T cells were magnetically purified from spleen and lymph node cells using FITC-conjugated anti-CD8β Abs. OT-I T cells (10^6) were CFSE-labeled and cultured with F4/80+ cells (2 × 10^5) and OVA257–264 peptide (10−10 M). Proliferation of OT-I T cells was determined by flow cytometry after 4 days of culture.

Cytokine secretion and intracellular cytokine staining
Peritoneal wash cells (10^6) from tumor-bearing mice treated with wtNKG2D or chNKG2D T cells were cultured in 48-well plates in complete medium. Twenty-four-hour cell-free conditioned media were 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) according to manufacturers’ protocols. Seventy-two-hour conditioned media were assayed for additional cytokines using multiplex analysis (Bio-Rad) by the Immune Monitoring Laboratory of the Norris Cotton Cancer Center (Lebanon, NH). For intracellular staining, peritoneal wash cells (10^6) or spleen cells (2.5 × 10^6) were cultured in complete medium for 24 h. Brefeldin A (10 μM) (Sigma-Aldrich) was added during the last 5 h of culture. Cells were fixed and stained with FITC-conjugated anti-CD8β (clone CT-CD8β), allophycocyanin-conjugated anti-NK1.1.1 (clone PK136), or allophycocyanin-conjugated anti-CD45.1.1 (clone A20) and biotin-conjugated anti-CD3 (clone eBio500A2), with a PE-Cy5.5 conjugated-streptavidin secondary. Cells were fixed with 1% paraformaldehyde, permeabilized with 0.1% saponin, and stained with PE-conjugated anti-IFN-γ (clone XMG12), or PE-conjugated anti-rat IgG1 isotype control.

Statistical analysis
Differences between groups were analyzed using the Student’s t test or ANOVA using Prism software (GraphPad Software). For survival studies, Kaplan-Meier curves were plotted and analyzed using the log rank test and Prism software. Values of p < 0.05 were considered significant.

Results

Treatment with chNKG2D T cells induced activation of the tumor-infiltrating leukocyte populations
To determine how treatment with chNKG2D T cells alters the tumor microenvironment, ID8 tumor cells were injected into B6 mice and mice were treated with chNKG2D or wtNKG2D T cells that were congenically marked with Ly5.1+1 after 1 week. Multiple host leukocyte populations were altered at the tumor site after chNKG2D T cell injection. The number of NK cells increased after chNKG2D T cell injection, with the peak response 3 days after T cell transfer (Fig. 1A). An increased percentage of NK cells expressed CD69, indicating the infiltrating NK cells were more activated in mice treated with chNKG2D T cells (Fig. 1B). The number of host Ly5.1+ CD8+ T cells in the peritoneal wash did not change after chNKG2D T cell treatment, however the host CD8+ T cells were more activated, as shown by an increased percentage of CD8+CD69+ cells (Fig. 1C). ChNKG2D T cell treatment also increased the number of GRI1+F4/80− cells, likely neutrophils, in the peritoneal wash (Fig. 1D). There was a significant difference in CD19+ B cells (Fig. 1E). chNKG2D T cells resulted in rapid decrease in the number of ID8 tumor cells just 1 day after T cell injection and it has previously been shown that chNKG2D T cells require perforin to directly kill ID8 tumor cells (Fig. 1F) (17). These data indicate that treatment with chNKG2D T cells induced a proinflammatory immune response at the tumor site, resulting in an infiltration and activation of immune cells that can decrease tumor burden, including NK cells and CD8+ T cells.

ChNKG2D T cells eliminate suppressive cells
CD4+ Treg cells can be found in ovarian tumors and have been previously shown to be present in mice bearing established ID8 tumors (8 weeks) (5, 7, 22). The effect of chNKG2D T cell treatment on the number of host Foxp3+CD4+ T cells in the tumor was determined. Mice with established tumors (7–9 weeks) were treated with wtNKG2D or chNKG2D T cells and the number of Foxp3+ T cells at the tumor site was determined 3 days after T cell transfer. Treatment with chNKG2D T cells decreased the number of Foxp3+CD4+ T cells at the tumor site by more than 300-fold compared with mice treated with wtNKG2D T cells or that received no treatment (Fig. 2A). CD8+Foxp3+ T cells were not detected in the peritoneal washes of the tumor-bearing mice (data not shown). Analyses with a soluble
NKG2D receptor showed that Foxp3⁺ CD4⁺ T cells from the tumor site expressed low levels of NKG2D ligands in all mice tested, indicating that these cells may be direct targets of chNKG2D T cells (Fig. 2B). Although Foxp3⁺ CD4⁺ T cells at the tumor site were eliminated, the number of Foxp3⁺ CD4⁺ T cells in the spleen was not altered after i.p. injection of chNKG2D T cells, suggesting that the depletion of regulatory cells was a local effect (Fig. 2C). Foxp3⁺ CD4⁺ T cells in the spleen of tumor-bearing mice or naïve mice did not express NKG2D ligands on the cell surface in all mice tested (Fig. 2D and data not shown), indicating that Treg cells increase expression of NKG2D ligands within the tumor environment.

To determine whether chNKG2D T cells were directly killing Foxp3⁺ CD4⁺ T cells, peritoneal wash cells from mice with established tumors (8–9 weeks) were cultured with wtNKG2D or chNKG2D T cells for 24 h (Fig. 2E). Culture with chNKG2D T cells resulted in much fewer live Foxp3⁺ CD4⁺ T cells compared with those cultured with wtNKG2D T cells or medium alone. ChNKG2D T cells required expression of perforin but not FasL to remove the Foxp3⁺ CD4⁺ T cells because there was no loss of Foxp3⁺ CD4⁺ T cells when they were cultured with chNKG2D T cells deficient in perforin, or with both perforin and FasL. These data are consistent with the idea that chNKG2D T cells directly kill tumor-associated Foxp3⁺ CD4⁺ T cells using perforin. However, this reduction in vitro was not as large as observed in vivo, suggesting that other mechanisms may also be involved to eliminate Foxp3⁺ CD4⁺ T cells in vivo.

In addition to Treg cells, other populations present in the peritoneal wash of mice with large ascites at 8–9 weeks included ID8 tumor cells (10–15%) and leukocytes, including CD11c⁺, F4/80⁺ GR1⁺, F4/80⁺ GR1⁺, and CD19⁺ B cells. The number of CD19⁺ cells also decreased in vivo when mice were treated with chNKG2D T cells. However, the percent of CD19⁺ cells did not decrease when cultured with chNKG2D T cells in vitro (Fig. 2F). Thus, the change in B cell numbers in vivo is likely not due to direct killing by chNKG2D T cells.

Treatment with chNKG2D T cells also induced changes in the APCs at the tumor site. There was an increase in CD11c⁺ MHC class II⁺⁺ dendritic cells (DCs) (Fig. 3A), chNKG2D T cells also altered the phenotype of the tumor-infiltrating macrophages. Macrophages in wtNKG2D T cell-treated mice expressed high levels of F4/80 and had low MHC class II expression. After chNKG2D T cell treatment, the macrophages decreased F4/80 expression and...
increased GR1 and MHC class II expression (Fig. 3B). Macrophages from both wtNKG2D and chNKG2D T cell-treated mice expressed CD11b. Although some previous studies have shown that tumor associated, GR1-expressing macrophages can have an immunosuppressive phenotype, other studies have shown that this is an activated cell phenotype (10, 23). To investigate whether chNKG2D T cells altered the function of macrophages to become immunostimulatory or immunosuppressive, F4/80

/H11001

cells from mice treated with wtNKG2D or chNKG2D T cells were isolated from peritoneal washes and cultured with CFSE-labeled OT-I T cells and OVA peptide. OT-I T cell proliferation was measured after 4 days of culture. The average of each group + SD (n = 4) is shown. Treatment with chNKG2D T cells significantly changed the number of the different cell populations compared with control-treated mice (+, p < 0.05). Data are representative of at least two separate experiments.

FIGURE 3. APCs at the tumor site are activated after chNKG2D T cell injection. ID8-GFP cells were injected i.p. into mice. After 7 days, mice were treated with wtNKG2D (▲) or chNKG2D (●) T cells i.p. The number of CD11c

/H11001

MHC class II

/high cells (A) or the number of F4/80

/GR1

cells (B) was determined before T cell injection (day 0), and 1, 3, and 7 days after T cell injection. C, F4/80

/cells were isolated from the peritoneal washes 3 days after injection of wtNKG2D (white bar) or chNKG2D (black bar) T cells or from naive mice (gray bar) and were cultured with CFSE-labeled OT-I T cells and OVA peptide. OT-I T cell proliferation was measured after 4 days of culture. The average of each group + SD (n = 4) (C) and representative histograms of OT-I T cell proliferation (D) are shown. Treatment with chNKG2D T cells significantly changed the number of the different cell populations compared with control-treated mice (+, p < 0.05). Data are representative of at least two separate experiments.

chNKG2D T cell treatment leads to production of IFN-γ and NO by tumor-infiltrating host cells

IFN-γ has multiple antitumor properties and previous studies have shown that chNKG2D T cells secrete IFN-γ when cultured with ID8 tumor cells in vitro and that IFN-γ is an important effector molecule for antitumor efficacy in vivo (17, 19). To determine whether chNKG2D T cell treatment induced IFN-γ production at the tumor site and cytokine production was measured in peritoneal cells isolated from tumor-bearing mice treated with wtNKG2D or chNKG2D T cells. Peritoneal wash cells from chNKG2D T cell-treated mice secreted more IFN-γ compared with cells isolated from wtNKG2D T cell-treated mice (Fig. 4A). Additionally, the peritoneal wash cells from chNKG2D T cell-treated mice secreted more NO (Fig. 4A), possibly due to the increased IFN-γ production as this cytokine can induce macrophage activation. The significantly increased cytokine response was observed as early as 1 day after chNKG2D T cell injection with a peak occurring 7 days after chNKG2D T cell injection.
after T cell injection. Intracellular staining for IFN-γ was performed to determine which cells at the tumor site were producing IFN-γ. Ly5.1+ chNK2G2D T cells secreted significant levels of IFN-γ 1 and 3 days after injection. However, after 7 days, the transferred T cells were no longer found in the peritoneal cavity (Fig. 4B). In addition to the transferred T cells, host NK cells, CD4+, and CD8+ T cells produced significantly more IFN-γ in chNK2G2D T cell–treated mice compared with mice treated with wtNK2G2D T cells. Host cell production of IFN-γ began 1 day after chNK2G2D T cell injection and this host immune response continued to increase for 7 days. This indicated that chNK2G2D T cells induced a host immune response at the tumor site.

Changes in the tumor-infiltrating populations require chNK2G2D T cell–derived cytokines and perforin

Expression of perforin, GM-CSF, and IFN-γ by chNK2G2D T cells is required for complete reduction in tumor burden (17, 18). As these effector molecules likely contribute to the activation of the host immune response, the requirement of these molecules for chNK2G2D T cell–induced changes at the tumor site was determined. Tumor-bearing mice were treated with wtNK2G2D or chNK2G2D T cells derived from B6 mice or mice deficient in GM-CSF, IFN-γ, or perforin. Three days after T cell injection, the tumor-infiltrating populations were measured (Table I). chNK2G2D T cell–derived GM-CSF was involved in inducing a significant increase in NK cells and NK cell activation, CD8+ T cell activation, DCs, and neutrophils, and a decrease in B cells, because mice treated with chNK2G2D T cells deficient in GM-CSF did not have these changes in these leukocyte populations.

ChNK2G2D T cell–derived IFN-γ was required for a significant increase in NK cells and in NK cell activation, CD8+ T cell activation, neutrophils, and activation of DCs and macrophages. ChNK2G2D T cell–derived IFN-γ was also required for the alteration in B cells because mice treated with chNK2G2D T cells deficient in IFN-γ did not have this decrease. To determine whether the chNK2G2D T cell–derived IFN-γ had a direct effect on host cells, ID8-GFP cells were injected into mice deficient in IFN-γR1 and the mice were treated with wtNK2G2D or chNK2G2D T cells. Similar to mice treated with IFN-γ–deficient chNK2G2D T cells, mice deficient in IFN-γR1 treated with chNK2G2D T cells did not have an increase in NK cells and NK cell activation, CD8+ T cell activation, neutrophils, or maturation and activation of DCs and macrophages. Thus, IFN-γ derived from chNK2G2D T cells acts on the cells of the host to induce changes in the tumor microenvironment. chNK2G2D T cell–derived perforin was required for the decrease in CD19+ cells, while all other changes in leukocytes were observed when mice were treated with perforin–deficient chNK2G2D T cells. However, CD19+ cells did not express NK2G2D ligands and were shown to not be direct targets of chNK2G2D T cells (Fig. 2F). chNK2G2D T cell–derived perforin was also required for the decrease in ID8 tumor cells.

The requirement of chNK2G2D T cell–derived molecules for the induction of cytokine secretion at the tumor site was also determined. Peritoneal cells had increased secretion of IFN-γ after injection of chNK2G2D T cells, and this increase was significantly diminished when chNK2G2D T cells lacked GM-CSF, IFN-γ, or perforin (Fig. 5A). Although all three effector molecules from chNK2G2D T cells were required for the induction of a host IFN-γ response, only chNK2G2D T cell–derived IFN-γ was required for production of NO at 3 and 7 days after T cell injection. Mice deficient in IFN-γR1 also did not have an increase in IFN-γ or NO secretion after treatment with chNK2G2D T cells, demonstrating that host cells need to be responsive to IFN-γ to increase production of these cytokines at the tumor site (Fig. 5B). Intracellular staining was performed to determine which of the IFN-γ–producing cells the chNK2G2D T cell–derived molecules affected. There were lower percentages of NK cells and CD4+ T cells secreting IFN-γ when chNK2G2D T cells were deficient in GM-CSF, IFN-γ, or perforin, indicating that all three molecules were involved in the induction of NK cell and CD4+ T cell production of IFN-γ (Fig. 5C). Although there was a reduced percentage of CD8+ T cells producing IFN-γ when chNK2G2D T cells were deficient in GM-CSF, IFN-γ, or perforin, this reduction was not significant.

The secretion of additional cytokines by peritoneal cells was altered after treatment with chNK2G2D T cells. Many proinflammatory cytokines were increased after chNK2G2D T cell treatment, including IL-1, IL-2, IL-12, CCL2, CCL3, and CCL5 (Table II). Anti-inflammatory cytokines IL-9 and IL-10 were decreased in chNK2G2D T cell–treated mice. The induction of these cytokines also required chNK2G2D T cell–derived molecules. chNK2G2D T cell–derived IFN-γ was essential for the production of IL-1, IL-2, IL-12, G-CSF, GM-CSF, CCL3, and CCL5, and also for decreasing the amount of IL-6. GM-CSF from chNK2G2D T cells had a similar role in cytokine induction and additionally increased CCL2 and decreased TNF-α production. chNK2G2D T cell–derived perforin was necessary for the increase in IL-1, G-CSF, CCL2, and CCL3. Cytokines that did not change after chNK2G2D T cell treatment included IL-3, IL-5, IL-13, IL-17, KC, and CCL4, and cytokines not found at the tumor site included IL-4 and eotaxin (data not shown). Together, these data demonstrate that treatment of tumor-bearing mice with chNK2G2D T cells induced a proinflammatory host immune response at the tumor site, decreased immunosuppressive regulatory cells and increased cell populations with antitumor capabilities and the local production of proinflammatory cytokines.

Table I. Leukocyte and tumor cell numbers at the tumor site 3 days after T cell injection

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* Significantly different from wtNK2G2D T cell–treated mice, p < 0.05.
† Significantly different from chNK2G2D T cell–treated mice, p < 0.05.
FIGURE 5. chNKG2D T cell-derived GM-CSF, IFN-γ, and perforin are required for the increase in IFN-γ and NO production in tumor-bearing mice. A, Peritoneal wash cells from tumor-bearing mice treated with B6-derived wtNKG2D (WT; □) or chNKG2D T cells (CH; ■), or chNKG2D T cells deficient in GM-CSF (□), IFN-γ (□), or perforin (Pfp; □) were cultured in medium for 24 h. Cell-free supernatants were assayed for IFN-γ or NO. B, Three days after T cell injection, peritoneal wash cells from B6 mice treated with wtNKG2D (□) or chNKG2D T cells (■), or IFN-γR1−/− mice treated with wtNKG2D (□) or chNKG2D T cells (■) were cultured in medium for 24 h. Cell-free supernatants were assayed for IFN-γ or NO. C, Seven days after T cell transfer, intracellular staining was performed on peritoneal wash cells cultured in medium for 24 h. Cells were evaluated for IFN-γ production and were gated on either CD8+CD3+CD4−CD11b− or NK1.1+CD3− as indicated. The average of each group + SD (n = 4) is shown. Treatment with chNKG2D T cells significantly increased IFN-γ and NO secretion compared with control-treated mice (*, p < 0.05) and mice treated with chNKG2D T cells deficient in effector molecules produced significantly less IFN-γ and NO compared with chNKG2D T cell-treated mice (§, p < 0.05). Data are representative of at least two separate experiments.

Treatment of advanced tumors with chNKG2D T cells induced activation of the tumor-infiltrating leukocyte populations and increased survival of tumor-bearing mice

Previous studies showed that treatment of ID8-tumor-bearing mice with chNKG2D T cells 1, 2, and 3 weeks after tumor cell injection lead to long-term, tumor-free survival in 100% of the mice (17). However, the efficacy of chNKG2D T cells treating mice with established solid tumors had not been tested before this study. First, it was determined whether chNKG2D T cells induced changes in the tumor microenvironment in mice bearing 5-week tumors, which is a time when many solid tumors have been established on the peritoneal wall. Compared with treating 1 week after tumor cell injection, chNKG2D T cell treatment induced similar changes in tumor-infiltrating populations in mice bearing tumors for 5 weeks, including an increase in the number of activated NK cells, CD8+ T cells, and macrophages; an increase in MHC class II+ DCs; an increase in neutrophils; and a decrease in B cells and in ID8-GFP tumor cells (Fig. 6). Similar changes were also seen after treatment with chNKG2D T cells in mice with tumors established for 7–9 weeks (data not shown). There was also an increase in IFN-γ and NO secretion from peritoneal wash cells in chNKG2D T cell-treated mice. This indicated that chNKG2D T cells led to the activation of the tumor-associated leukocytes even in mice bearing tumors established for 5 weeks.

To determine whether treatment with chNKG2D T cells could increase survival in mice with established solid tumors, wtNKG2D or chNKG2D T cells were transferred to tumor-bearing mice 5, 6, and 7 weeks after tumor cell injection (Fig. 7A). Although mice treated with wtNKG2D T cells had a median survival of 88 days, treatment with chNKG2D T cells significantly increased the survival of tumor-bearing mice. All chNKG2D T cell-treated mice survived longer than the mice treated with wtNKG2D T cells, and seven of 12 chNKG2D T cell-treated mice survived long-term and were tumor-free 225 days after tumor cell injection. The chNKG2D T cell-treated mice had large tumor burdens at the time of death, with all mice having over 100 solid tumors on the peritoneal cavity. However, the five chNKG2D T cell-treated mice that were killed due to tumor growth had fewer solid tumors on the peritoneal cavity, ranging from 8 to 40 large solid tumors. This indicated that while some chNKG2D T

Table II. Cytokine secretion by peritoneal cells 3 days after T cell injection

<table>
<thead>
<tr>
<th>T cell donor</th>
<th>WT</th>
<th>CH</th>
<th>GM-CSF+/−</th>
<th>CH</th>
<th>IFN-γ+/−</th>
<th>CH</th>
<th>Pfp+/−</th>
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<tr>
<td>IL-1α</td>
<td>7</td>
<td>156†</td>
<td>13†</td>
<td>4†</td>
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<tr>
<td>IL-1β</td>
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<td>41†</td>
<td>15†</td>
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<tr>
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<td>561†</td>
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<td>902†</td>
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<td>95†</td>
<td>34†</td>
<td>31†</td>
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<td>IL-12 p60</td>
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<td>66†</td>
<td>108†</td>
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<tr>
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<td>23†</td>
<td>4†</td>
<td>4†</td>
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<td></td>
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</tbody>
</table>

* Values shown are pg/ml, averages of three mice per group. WT, wtNKG2D T cell-treated mice; CH, chNKG2D T cell-treated mice; Pfp, perforin.
† Values were above detection limit of assay.
§ Significantly different from wtNKG2D T cell-treated mice, p < 0.05.
cell-treated mice could not control tumor growth, their tumor burden was still decreased compared with wtNKG2D T cell-treated mice.

Transferring chNKG2D T cells at weekly intervals may not be an ideal therapeutic approach, as the peak of the IFN-γ immune response occurred 1 week after chNKG2D T cell injection. Increasing the second dose of chNKG2D T cells during the peak of the immune response from the first chNKG2D T cell injection may not result in a stronger boost in the ongoing immune response. Therefore, the timing of the chNKG2D T cell injections was altered to be administered 5, 7, and 9 weeks after tumor cell injection (Fig. 7B).

Using this treatment regimen, transfer of chNKG2D T cells led to long-term, tumor-free survival in 100% of the mice bearing tumors for 5 weeks. This demonstrates that successful treatment of established tumors did not require the administration of more chNKG2D T cells but an optimal timing of the T cell infusion based on what occurred in the microenvironment was necessary.

**Discussion**

Therapies that alter leukocyte populations in the tumor microenvironment to decrease the effect of the immunosuppressive populations and induce the recruitment and activation of antitumor immune cells may lead to the development of long-lived anti-tumor immune responses and improved cancer therapy. These data show that treatment with chNKG2D T cells induced a proinflammatory immune response at the tumor site in a mouse model of ovarian cancer. chNKG2D T cell treatment led to an increase in activated NK cells and activated CD8+ T cells, and a decrease in Foxp3+ Treg cells. Tumor-infiltrating macrophages and DCs also became activated after chNKG2D T cell treatment, increasing their activation of Ag-specific T cells. Cells isolated from the peritoneal cavity produced increased amounts of IFN-γ, NO, and other proinflammatory cytokines. These changes in tumor-infiltrating populations and cytokine secretion required chNKG2D T cell treatment and improved cancer therapy. These data show that treatment with chNKG2D T cells can express PD-1 and interaction with PD-L1 and PD-L2 expressed by cells at the tumor site inhibits T cell proliferation, cytokine secretion, and cytotoxicity (8, 26, 27). Tumor-infiltrating cells but an optimal timing of the T cell infusion based on what occurred in the microenvironment was necessary.

Many immunosuppressive cells and molecules are found in advanced ovarian cancer, including myeloid-derived suppressor cells and Treg cells. These cells can inhibit the immune response to ovarian cancer cells through multiple mechanisms. Tumor-associated myeloid cells may express molecules that can inhibit T cell responses, including B7-H1 and B7-H4 (8, 24, 25). Tumor-infiltrating T cells can express PD-1 and interaction with PD-L1 and PD-L2 expressed by cells at the tumor site inhibits T cell proliferation, cytokine secretion, and cytotoxicity (8, 26, 27). Additionally, tumor-associated macrophages may express IDO, PGE2, and...
arginase, which may inhibit T cell responses through down-regulating CD3 expression, inhibiting T cell proliferation, or inducing apoptosis (9, 10, 28). Although many studies suggest that CD11b+ GR-1+ myeloid cells are suppressive in the tumor microenvironment, other studies in infection models find that GR-1-expressing macrophages are inflammatory monocytes (9, 10, 23). These F4/80+ GR-1+ inflammatory monocytes secrete cytokines, including NO, IL-12, and TNF-α, and are involved in clearance of infections such as *Listeria monocytogenes* and *Toxoplasma gondii* (23, 29, 30). Treatment of tumor-bearing mice with chNK2G2 T cells changes the phenotype of the tumor-associated macrophages, such that the cells display characteristics of inflammatory macrophages, expressing GR-1, and secreting NO. These cells also stimulated Ag-specific T cell proliferation. Thus, chNK2G2 T cells reversed the immunosuppressive phenotype of tumor-associated macrophages to become proinflammatory, tipping the balance in favor of developing a host immune response against the tumor. Activation of macrophages required chNK2G2 T cell-derived IFN-γ and host cell responsiveness to IFN-γ, demonstrating that expression of IFN-γ is essential for alteration of macrophages at the tumor site.

Foxp3+ Treg cells are found in human and murine ovarian tumors and the presence of Treg cells is inversely correlated with survival (31, 32). Treg cells isolated from ovarian cancer ascites samples can inhibit the proliferation, cytokine secretion, and cytotoxicity of tumor-infiltrating T cells (5, 31). chNK2G2 T cell treatment of established tumors almost completely eliminated Foxp3+ Treg cells at the tumor site. It has been shown that human adaptive Treg cells can express NKG2D ligands during infection with *Mycobacterium tuberculosis*, but that natural Treg cells did not express NKG2D ligands (33). Similarly, this study showed that murine Foxp3+ CD4+ Treg cells isolated from the tumor environment expressed NKG2D ligands, while Treg cells from the spleen of tumor-bearing mice or from naive mice did not express NKG2D ligands on their cell surface. Thus tumor-associated Treg cells are potential direct targets for chNK2G2 T cells and chNK2G2 T cells were shown to kill Treg cells in vitro through a mechanism that required expression of perforin but not FasL. The elimination of this suppressive population from the tumor site would allow tumor-infiltrating immune cells to be more effective.

A large proportion of the cells at the tumor site were CD19+ B cells and this population was also altered after chNK2G2 T cell treatment. These cells can express PD-L1 and PD-L2 and can secrete anti-inflammatory cytokines including IL-10 that may inhibit immune responses to tumors (34, 35), chNK2G2 T cells required the expression of not only GM-CSF and IFN-γ, but also perforin for the decrease in B cells. IFN-γ and TLR stimulation can induce the egress of B cells from the peritoneal cavity, thus IFN-γ secretion from chNK2G2 T cells and host cells may directly cause the trafficking of the B cells out of the peritoneal cavity (36, 37). Another possibility is that chNK2G2 T cells lysed the B cells; however, we did not detect NKG2D ligand expression on these B cells, nor did we observe killing of B cells in vitro, so direct killing was unlikely in vivo. Another hypothesis is that upon chNK2G2 T cell lysis of the ID8 tumor cells, endogenous molecules that can stimulate TLRs were released from the dying tumor cells, such as heat shock proteins (38). These molecules may stimulate TLRs on the B cells, causing their activation and subsequent trafficking from the peritoneal cavity.

In addition to decreasing immunosuppressive populations at the tumor site, chNK2G2 T cell treatment also increased cells that can potentially attack tumor cells. NK cells and CD8+ T cells both can lyse tumor cells, thus decreasing tumor burden and also releasing tumor Ags to promote Ag presentation. There was also an increase in the secretion of proinflammatory cytokines, including IFN-γ, by host cells at the tumor site. IFN-γ has many antitumor properties, including increasing Ag presentation, maturing APCs, decreasing angiogenesis, having cytostatic effects directly on tumor cells, and IFN-γ expression in human ovarian cancer is associated with a favorable prognosis (39–43). Components of the endogenous immune system may be involved in immune surveillance against the tumor even without chNK2G2 T cell treatment. Previous work has shown that the host immune system responds to this tumor, but this response is not sufficient for tumor elimination (44). Specifically, wtNK2G2 T cell-treated mice that were deficient in IFN-γ or NK cell depleted had greater tumor growth compared with B6 mice. This indicates that these host mechanisms play a role in controlling tumor growth; however, these host mechanisms are not able to eliminate the tumor, which may be due to immune suppression at the tumor site. chNK2G2 T cells likely act in combination with host immune cells to overcome local immune suppression and result in tumor elimination. Additional work has shown that host-derived perforin, IFN-γ, NK cells, and lymphocytes are all required for complete tumor reduction by chNK2G2 T cells (44). This further indicates the importance of the activation of the host immune cells for chNK2G2 T cells antitumor efficacy. Data show that host cells need to express IFN-γR1 for the immunostimulatory response at the tumor site, indicating that IFN-γ produced at the tumor site by chNK2G2 T cells and host cells is acting directly on host cells. One possible action may be to activate tumor-associated macrophages, thus changing the phenotype of the macrophages and inducing cytokine secretion. The activated macrophages may be secreting NO, which can have anti-tumor effects including direct killing of ID8 tumor cells, or may be secreting other cytokines that activate host NK cells and T cells (45–47).

Through developing a better understanding of the immune response generated by the chNK2G2 T cells at the tumor site, this study helped to develop a treatment regimen that was successful at treating tumors established for 5 weeks, a time when many solid tumors are well-established on the peritoneal wall. Instead of administering an increased number of chNK2G2 T cells to achieve long-term tumor-free survival in all mice, we altered the scheduling of the chNK2G2 T cell doses such that the second and third doses did not coincide with the peak of the ongoing host immune response after the initial chNK2G2 T cell injection. The treatment regimen of administering chNK2G2 T cells every other week led to long-term tumor-free survival in 100% of mice. This illustrates that one may need to analyze the kinetics and type of immune response induced by the transferred T cells and determine the best therapeutic regimen to obtain optimal efficacy.

In this study, chNK2G2 T cells were able to reduce immunosuppressive cells and induce activation of host antitumor immune cells both in early and established tumors. This indicates that despite the increased prevalence of immunosuppressive cells in established tumors, chNK2G2 T cells may potentially be able to induce immune responses in patients with early or late stage tumors. chNK2G2 T cell-derived IFN-γ and GM-CSF were required for many of the changes in the leukocyte populations and for the secretion of proinflammatory cytokines. Previous work has shown that chNK2G2 T cell-derived IFN-γ and GM-CSF were also required for complete antitumor efficacy, indicating that changing the tumor microenvironment and inducing a proinflammatory response at the tumor site is essential for cancer therapy (17, 18). chNK2G2 T cells may lyse tumor cells, thus decreasing tumor burden and increasing Ag presentation. Additionally, chNK2G2 T cells reduce the immunosuppressive populations while concurrently secreting proinflammatory cytokines, which can recruit and activate host NK cells, T cells, and APCs. Although many current studies need to combine multiple different therapies to decrease immunosuppression and increase the activation of the immune response, this study shows
Acknowledgments

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disorders highly represented in ovarian cancer.


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.