Cancer Immunotherapy Using a Bispecific NK Receptor Fusion Protein that Engages both T Cells and Tumor Cells

Tong Zhang and Charles L. Sentman

Abstract

T-cell immunotherapy is a promising strategy to treat cancer, but its efficacy, complexity, and costs may pose challenges. In this study, we report the results of an investigation of a new approach to selectively activate a T-cell attack against tumor cells. The immunotherapeutic approach we developed utilizes a bifunctional fusion protein that binds tumor cells through NK (natural killer)–activating receptor NKG2D and that recruits and stimulates T cells through an anti-CD3 single-chain variable fragment (scFv-NKG2D). In vitro, this scFv-NKG2D fusion protein engaged both T cells and tumor cells, stimulating T cells to produce IFN-γ, and cytotoxicity against NKG2D ligand–positive tumor cells. In vivo, expression of scFv-NKG2D by NKG2D ligand–positive tumor cells reduced tumor burden and, in some cases, led to tumor-free survival. Administration of scFv-NKG2D in vivo also promoted survival in a murine lymphoma model. Tumor-free mice were resistant to rechallenge with cognate tumor cells, suggesting that a host-specific immunologic memory response had been generated. Host adaptive immunity including γβ T cells was required for scFv-NKG2D–mediated therapeutic efficacy. ScFv-NKG2D also inhibited the growth of NKG2D ligand–negative B16F10 tumors, reduced the percentage of myeloid-derived suppressor cells and regulatory T cells, and increased the infiltration of T cells, suggesting that scFv-NKG2D may target these immune suppressive cells. Together, these results establish scFv-NKG2D as a promising biological fusion protein to induce effective antitumor immunity. Cancer Res; 71(6); 2066–76. ©2011 AACR.
T cells against multiple types of tumor cells and local tumor immune suppressor cells.

**Materials and Methods**

**Mice and cell lines**

C57BL/6 [B6; wild type (WT)] mice were purchased from National Cancer Institute (NCI). TCRα(−/−) mice (B6 background) were obtained from Dr. William R. Green (Dartmouth Medical School), Rag1(−/−) mice (B6 background) were purchased from the Jackson Laboratory and bred in the animal facility of Dartmouth College. Animals used in experiments were between 7 and 12 weeks of age. All experiments were conducted according to protocols approved by Dartmouth College’s Institutional Animal Care and Use Committee. Anti-mouse CD3ε hybridoma 145-2C11 was obtained from American Type Culture Collection (ATCC). H-2Kb-restricted B3Z T-cell hybridoma cells which recognize the OVA257-264 epitope were obtained from Dr. Nilabh Shastri (University of California at Berkeley). On activation, B3Z cells express the LacZ gene (12). Murine colon cancer MC-38 cells (H-2b) were obtained from Dr. Richard J. Barth (Dartmouth Medical School). Mouse T-cell line lymphoma RMA and RMA/RG, ovarian cancer cells ID8, and melanoma B16F10 have been described previously (13–15). Mastocytoma cell line P815/Rae1 was generated by retroviral transduction of P815 cells (H-2a) with a mouse NKG2D ligand, Rae1. RMA and B16F10 are NKG2D ligand-negative cells, whereas RMA/rag, P815/Rae1, ID8, and B3Z cells are NKG2D ligand-positive cells. Packaging cells PT67 (ATCC) and ovarian cancer B16F10 cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) with a high glucose concentration (4.5 g/L) supplemented with 10% heat-inactivated FBS (Atlanta Biologicals), 100 U/mL penicillin, 100 µg/mL streptomycin, 1 mmol/L pyruvate, 10 mmol/L HEPES, 0.1 mmol/L nonessential amino acids, and 50 µmol/L 2-mercaptoethanol. All other cell lines were cultured in RPMI plus the same supplements as in DMEM.

**Construction of scFv-NKG2D**

Variable regions of both heavy [VH, amino acids (aa) 1–136] and light chains (VL, without signal peptide, aa 21–128) were PCR amplified using cDNA derived from 145.2C11 hybridoma. Extracellular domain of murine NKG2D was amplified using the full-length NKG2D cDNA as a template (16). To make anti-CD3ε scFv, Vh and Vl were joined with a 15-amino acid glycine (G)–serine (S) linker (G4S3) (3 repeats of GGGGS). All PCR reactions were conducted using a high-fidelity DNA polymerase Phusion (New England Biolabs). All oligos were synthesized by either Integrated DNA Technologies or Sigma-Genosys. ScFv-NKG2D was created by joining anti-CD3ε scFv to the extracellular domain of mouse NKG2D (aa 90–232) with a second (G4S) linker. A histidine tag (6xHis) was added at the C-terminus to facilitate protein purification. The fusion gene was then cloned into a retroviral vector pMB-neo (Stratagene). A negative control fusion gene scFv-HuNKG2D was constructed by joining the scFv with the extracellular domain of human NKG2D gene.

**Production of scFv-NKG2D protein**

ScFv-NKG2D proteins were expressed in retroviral vector stably transduced B16F10 cells according to our previous protocols (16, 17). Briefly, B16F10 cells were retrovirally transduced with vectors that were generated from scFv-NKG2D stably transfected packaging cell PT67 for 5 rounds. Stable B16F10 cell lines with amplified expression of scFv-NKG2D (either human or mouse version) were selected with G418 (1.5 mg/mL) for 14 days. The resulting stable B16F10 lines (B16F10/scFv-mNKG2D and B16F10/scFv-HuNKG2D) were then cultured in serum-free media (293 SFM II; Invitrogen). Supernatants were collected every 48 hours and were subjected to affinity chromatography using HisTrap columns (GE Healthcare Bio-Sciences) according to the manufacturer’s instructions. Eluted fractions were then concentrated and desalted using Amicon Ultra columns (30k MWCO; Millipore). Purified scFv-NKG2D proteins were resuspended in PBS, filtered (pore size: 0.22 µm), and stored in −20°C. The integrity of scFv-NKG2D protein was determined by SDS-PAGE, followed by staining with SYPRO orange (Invitrogen), and visualized using a Typhoon 9400 imager (GE Healthcare). Concentration of scFv-NKG2D was quantitated with ImageJ software (NIH; http://rsb.info.nih.gov/ij/image/Default.html).

**Flow cytometry**

To determine whether scFv-NKG2D binds to CD3ε, RMA cells were stained with scFv-NKG2D (0.01–1 µg/mL), followed by staining with phycoerythrin (PE)-labeled anti-mouse NKG2D monoclonal antibody (mAb) (CX5; ebioScience) or isotype control mAb. In a blocking experiment, RMA cells were preincubated with anti-CD3ε (0.01–1 µg/mL; 145.2C11, ebioScience) at room temperature for 15 minutes before staining with scFv-NKG2D. Rae1 expression was determined by flow cytometry by using APC labeled pan-anti-Rae1 mAb (clone 186107; R&D Systems). Infiltration of CD4+ and CD8+ T cells, T-cell activation (CD69 expression), MDSCs (CD11b+ Gr1+), and Tregs (CD4+ Foxp3+) in tumors were determined by flow cytometry after digestion of excised, established tumors by using cocktails of DNase and collagenase according to our previous protocol (18). All samples were preincubated with FcR block antibody (anti-mouse CD16/CD32) to reduce nonspecific staining. Cell fluorescence was monitored using an Accuri C6 flow cytometer. Flow cytometric analysis was carried out using either Accuri or Flowjo software.

**Cytokine production by T cells**

To determine whether scFv-NKG2D can engage both T cells and tumor cells and lead to T-cell activation, spleen cells were stimulated with ConA and IL-2 for 4 days before coculture with tumor cells with or without scFv-NKG2D (50 ng/mL) for 24 hours. Amounts of IFN-γ in supernatants were analyzed with ELISA. For tumor cells grown in suspension, coculture with preactivated murine primary T cells (107) was done in round-bottom 96-well plates at a ratio of 1:1, whereas adherent tumor cells (2.5 × 106) were cocultured with T cells in flat-bottom plates. Tumor cells were irradiated (120 Gy) before use. Cell-free supernatants were collected after 24 hours.
Twenty-four hour supernatants were assayed for IFN-γ by ELISA, using DuoSet ELISA kits (R&D Systems).

**Cytotoxicity assay**

Lysis of target cells was determined by a 5-hour 51Cr release assay as previously described (16, 17).

**BetaRed staining**

The LacZ activity in B3Z cells was determined by using a BetaRed β-galactosidase assay kit (EMD Bioscience) according to the manufacturer’s instruction.

**Tumor inoculation**

For the determination of effects of local expression of scFv-NKG2D on MC-38 or B16F10 tumor growth, either WT or scFv-NKG2D–transduced tumor cells (5 × 10⁵) were injected subcutaneously into the shaved right flank of recipient mice. Tumors were then measured every 2 days with a caliper and tumor areas were calculated. Mice were sacrificed when tumor burden became excessive. Animals were regarded as moribund when no tumor was found 4 weeks after inoculation. For the rechallenge experiments, mice were inoculated with 10⁵ WT MC-38 cells on the shaved left flank. In the systemic lymphoma model, B6 mice were injected with 10⁵ RMA/RG cells via tail veins in 400 μL of HBSS. For treatment with recombinant scFv-NKG2D proteins (either mouse or human NKG2D fusion protein), mice were administered intravenously with 5 μg scFv-NKG2D on days 5, 7, and 9 post–tumor inoculations. Mice were monitored closely and sacrificed when moribund signs were observed.

**Statistical analysis**

Differences between groups were analyzed using Student’s t test. Values of P < 0.05 were considered significant. Kaplan–Meier survival curves were plotted and analyzed using the GraphPad Prism software (GraphPad Software).

**Results**

**Construction and expression of ScFv-NKG2D**

ScFv-NKG2D was constructed by joining anti-CD3ε scFv to the extracellular domain of NKG2D receptor. The structure of ScFv-NKG2D is shown in Figure 1. Two 15-aa (G4S)₃ linkers were inserted between VH and VL and between scFv and NKG2D to allow flexible interactions among scFv-NKG2D, CD3ε, and NKG2D ligands. Human NKG2D was also used to make a control fusion protein scFv-HuNKG2D because human NKG2D receptor has minimal cross-reactivity with mouse NKG2D ligands. In scFv, the antibody Fc fragment is eliminated, resulting in less nontumor associated T-cell activation. As shown in Figure 1B, purified scFv-NKG2D proteins are monomers at sizes around 50 kD under both native and reducing conditions, which were in line with the expected sizes of 44 kD.

**ScFv-NKG2D fusion protein binds to both CD3ε and NKG2D ligands**

To determine whether scFv-NKG2D fusion protein preserves the binding affinity and specificity of the parental anti-CD3ε 145-2C11 antibody, CD3ε-NKG2D T-cell lymphoma RMA cells were stained with scFv-NKG2D (0.01–1 μg/mL), followed by staining with anti-NKG2D-PE (Fig. 2A). As shown in Figure 2B, scFv-NKG2D binds to CD3ε in a dose-dependent manner. In addition, preincubation of RMA cells with 145-2C11 mAb (0.1–10 μg/mL) could block the binding of the scFv-NKG2D fusion protein to RMA cells in a dose-dependent manner, indicating the specific recognition of CD3ε molecule by scFv-NKG2D (Fig. 2C).

To examine whether scFv-NKG2D was capable of binding simultaneously to both T cells via scFv and tumor cells via NKG2D (Fig. 2D), a mouse T hybridoma B3Z was used. B3Z T cells express both CD3ε and an NKG2D ligand Multi1 (data not shown). We hypothesized that if scFv-NKG2D binds to CD3ε and Multi1 on B3Z cells simultaneously, cross-linking of CD3ε molecule will lead to activation of T cells. Because B3Z cells contain the IL-2 promoter–controlled LacZ gene as a reporter, after activation, B3Z cells will express LacZ (β-galactosidase). The ability of scFv-NKG2D to stimulate Multi1⁺ B3Z cells was evaluated by measurement of the OD₅₅₀ nm or OD₅₉₀ nm values after addition of a BetaRed substrate. As expected, incubation...
of B3Z cells with scFv-NKG2D, but not with control molecule scFv-HuNKG2D (human), led to cell activation in a dose-dependent manner, indicating that the scFv-NKG2D can engage both T and tumor cells via NKG2D ligands. On the basis of data (Fig. 2D), we estimate the ED50 value of scFv-NKG2D for activating T cells was about 10 ng/mL. ScFv-NKG2D activates primary T cells by producing IFN-γ and exerting cytotoxicity.

To investigate whether scFv-NKG2D stimulates primary T cells, we examined IFN-γ production and cytotoxicity of T cells against tumor cells in the presence of scFv-NKG2D. ConA and IL-2 (25 U/mL) preactivated T cells were used as effector cells. As shown in Figure 3A and B, in the presence of scFv-NKG2D (50 ng/mL), T cells produced significant amounts of IFN-γ after coculture with NKG2D ligand–positive cells (RMA/Rae1, P815/Rae1, and ID8) but not with ligand-negative RMA and B16F10 cells, indicating that scFv-NKG2D allows T cells to functionally recognize NKG2D ligand–bearing tumor cells. In contrast, control protein scFv-HuNKG2D does not significantly stimulate T cells to produce IFN-γ in the presence of NKG2D ligand–positive tumor cells. These data indicate that anti-CD3ε scFv itself has minimal ability to activate T cells under these conditions. T-cell cytotoxicity in the presence of scFv-NKG2D against various tumor cells was also determined. As shown in Figure 3C, T cells could lyse NKG2D ligand–positive target cells (P815/Rae1, ID8, and MC-38) but not the ligand-negative cell line B16F10 in vitro in the presence of scFv-NKG2D. Similar to IFN-γ production, no significant killing was observed when control protein scFv-HuNKG2D was used. In addition, we also showed that scFv-NKG2D did not affect NK cell–mediated cytotoxicity against tumor cells despite the fact that incubation of high concentration (1 mg/mL) of scFv-NKG2D with tumor cells reduced surface NKG2D ligand expression (Supplementary Fig. S1).

**ScFv-NKG2D suppresses growth of NKG2D ligand–positive tumor cells in vivo and induces a host memory response**

Having shown that T cells could react against NKG2D ligand–positive tumor cells in vitro after engagement with scFv-NKG2D, we determined the therapeutic potential of scFv-NKG2D in vivo. First, colon cancer MC-38 cells that stably secreted scFv-mNKG2D (Supplementary Fig. S2) were
tested for growth in vivo after subcutaneous injection. This design represents a localized scenario for scFv-NKG2D treatment that could be produced by exogenous gene transfer. MC-38 cells expressing scFv-HuNKG2D fusion protein were used as the negative control. There was no significant difference between WT and scFv-NKG2D–expressing MC-38 cells to grow in vitro (data not shown). As shown in Figure 4A, scFv-mNKG2D expression by MC-38 tumor cells significantly reduced or prevented tumor growth compared with WT and scFv-HuNKG2D–expressing MC-38 tumor cells. Fifty-nine percent of B6 mice (13/22) receiving MC-38/scFv-mNKG2D tumor cells were tumor-free after 30 days. In a second and more stringent model, lymphoma-bearing mice that had been given NKG2D ligand–positive T-cell lymphoma RMA/RG cells intravenously were treated with 3 doses of either recombinant scFv-mNKG2D (5 µg per dose i.v.) or scFv-HuNKG2D (specificity control). As shown in Figure 4B, systemic administration of scFv-mNKG2D significantly improved survival of tumor-bearing mice. The median survival increased from 18 days (HBSS) to 31 days (P < 0.01), whereas treatment with control molecule scFv-HuNKG2D lead only to median survival of 23 days. In addition, 39% (7/18) of the scFv-mNKG2D–treated lymphoma-bearing mice were tumor free after 45 days. As for toxicity, the animals treated with scFv-mNKG2D proteins did not show any overt evidence of inflammatory damage (e.g., ruffled hair, hunchback, or diarrhea), suggesting there was no overt toxicity (data not shown).

To determine whether treatment with scFv-NKG2D induced a host memory response against WT tumor cells, scFv-NKG2D–treated mice that had remained tumor free after 45 days were challenged with either WT MC-38 (10³) or RMA (NKG2D ligand-negative, 10³) cells on their left flank. These tumor-free mice were resistant to a subsequent challenge of the same type of tumor cells, whereas all control naive mice had large tumors after 3 weeks (Fig. 4C and D). This observation indicated that scFv-NKG2D–treatment induced hosts to generate specific immunologic memory against tumor antigens.
Host adaptive immunity is involved in scFv-NKG2D–mediated antitumor efficacy

Because scFv-NKG2D protein uses T cells to target tumor cells, it is expected that the absence of T cells would render scFv-NKG2D treatment ineffective in reducing tumor growth. To test this hypothesis, either WT MC-38 or scFv-NKG2D–expressing MC-38 cells (MC-38/scFv-NKG2D) were inoculated (at a dose of $5 \times 10^5$ cells) subcutaneously into B6.RAG1−/− mice, which lack T and B lymphocytes. As expected, both WT and MC-38/scFv-NKG2D cells grew progressively in B6.RAG1−/− mice (Fig. 5A). Thus, lymphocytes play an obligate role in scFv-NKG2D–mediated antitumor growth in vivo.

γδ T cells constitute a major T-cell subset in skin and intestine and can be harnessed for cancer immunotherapy (19). Therefore, scFv-NKG2D may mobilize and activate γδ T cells against subcutaneous tumors, resulting in tumor reduction. The role of γδ T cells was investigated by inoculating either WT MC-38 or scFv-mNKG2D–expressing MC-38/scFv-NKG2D cells into TCRα−/− mice, in which ab T cells are deficient and γδ T cells remain intact (20). MC-38 cells that express scFv-NKG2D show markedly slower growth than those that express WT tumors (Fig. 5A). However, no tumor-free mice were obtained. This result suggests that γδ T cells play a role in scFv-NKG2D–mediated therapeutic efficacy against subcutaneous tumors but ab T cells are also required for efficient antitumor effects.

Tumor-associated host cells play roles in scFv-NKG2D–mediated antitumor efficacy

Several studies have shown that some immune cells, such as Tregs and MDSCs express NKG2D ligands (10). Nausch and colleagues showed that CD11b⁺ F4/80⁺ Gr1⁺ MDSCs isolated from tumor-bearing mice but not myeloid cells from naive mice expressed an NKG2D ligand, Rae1. Interestingly, these tumor-associated MDSCs can stimulate NK cells to produce IFN-γ in a manner that is partially dependent on the interaction between NKG2D and its ligands (10). In fact, NKG2D can be used to target Tregs at the tumor site in ovarian cancer (11). To determine whether tumor-associated host cells are involved in scFv-NKG2D–mediated therapeutic efficacy, we tested the effects of local expression of scFv-NKG2D on the growth of B16F10 tumors, which do not express NKG2D ligands in vitro or in vivo (Supplementary Fig. S3). If B16F10 tumor–associated host cells (such as MDSCs and Tregs) are
targeted by scFv-NKG2D, it would be expected that B16F10 tumor growth may be reduced by the local expression of scFv-NKG2D. As shown in Figure 5B, local expression of scFv-NKG2D significantly reduced B16F10 tumor growth, suggesting that scFv-NKG2D may target ligand-positive, tumor-associated host cells. There was no difference between B16F10 and B16F10/scFv-NKG2D in terms of in vitro growth (Supplementary Fig. S4).

To further understand whether Tregs and MDSCs can be targeted, we first checked the NKG2D ligand Rae1 expression on those cells in both MC-38 and B16F10 models. As shown in Supplementary Figure S5C, Rae1 was expressed on tumor-infiltrated host cells. Next, it was tested whether local expression of scFv-NKG2D could alter the ratios of effector CD4⁺/Treg and Gr1⁻/Gr1⁺ (CD11b⁺/F4/80⁻/Gr1⁻). In contrast, CD4⁺/F4/80⁻/Gr1⁻ cells from naive B6 spleens did not express Rae1, suggesting that tumor microenvironment plays a role in NKG2D ligand expression. To test the hypothesis, WT B16F10 or B16F10/scFv-NKG2D cells (5×10⁵ cells) were injected into the right flanks of B6 mice. The infiltration of T cells (both CD4⁺ and CD8⁺) and activation of T cells, as shown by expression of an activation marker CD69⁺, were determined by flow cytometry 19 days post-tumor inoculation. As shown in Figure 7A and C, the percentage of both CD4⁺ and CD8⁺ T cells was significantly increased in the presence of scFv-NKG2D. In addition, T cells (especially CD8⁺ T cells) had a significantly increased expression of CD69, which was consistent with an activated status (Fig. 7B and D).

**Discussion**

Immunotherapy has emerged as a promising method to treat cancers due to the ability to selectively eliminate tumors. Both vaccination and adoptive immune cell transfer have been utilized against cancers (3). DC-based vaccines have been tested in both animal models and clinical trials. However, DCs need to be delicately conditioned with toll-like receptor agonists and cytokines to ensure delivery of both primary and costimulatory signals for T-cell stimulation (21). Otherwise, T cells may be induced into a tolerant state. Although DC-based immunotherapy has proven clinically safe and efficient to induce tumor-specific immune responses, only a limited number of objective clinical responses have been reported in cancer patients (22). In the second strategy, adoptive transfer of T cells or allogeneic bone marrow cells can be carried out (23). However, the traditional approaches for obtaining large numbers of tumor-specific T cells are difficult. Allogeneic bone marrow transplantation can cause severe acute graft-versus-host disease, and response rate is less than 15% (23). Compared with these 2 strategies, scFv-NKG2D no longer relies on generation of specific T cells or a regular

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**Figure 5.** Host adaptive immune cells are involved in scFv-NKG2D-mediated tumor rejection and local expression of scFv-NKG2D also reduces NKG2D ligand-negative tumor growth. A, either WT (circle) or scFv-NKG2D-expressing (square) MC-38 cells were injected subcutaneously (5×10⁵) into right flanks of Rag1⁻/⁻ (unfilled) or TCR post-expressing (filled) mice on day 0. Error bars, SEM of 3 pooled independent experiments. Local expression of scFv-NKG2D significantly (P<0.05 at days 5–19) suppressed the MC-38 tumor growth in TCR post expressing but not Rag1⁻/⁻ mice. B, either WT (+; n=10) or scFv-NKG2D-expressing (□; n=17) B16F10 cells were injected subcutaneously (5×10⁵) into right flanks of B6 mice on day 0. The tumor areas are represented as mean ± SEM. Error bars, SEM. Local expression of scFv-NKG2D significantly (P<0.01, at days 5–17) suppressed B16F10 tumor growth.
antigen presentation by DCs. scFv-NKG2D stimulates host T cells in an MHC-independent manner. Therefore scFv-NKG2D is unlikely to be affected by well-documented tumor-escape strategies, such as "downregulation" of MHC expression. Unlike most of other bispecific molecules in which a specific tumor-targeting scFv has to be made for targeting certain types of tumors, scFv-NKG2D is a pan-tumor—targeting reagent because NKG2D ligands are expressed on many types of tumor cells (colon, ovarian, breast, prostate, melanoma, leukemia, and lymphoma, among others). In addition, NKG2D ligands have restricted expression in normal tissues (9), but they are also expressed on immunosuppressive cells within the tumor microenvironment.

In this study, scFv-NKG2D treatment reduced or abrogated the growth of NKG2D ligand—positive tumors, suggesting that scFv-NKG2D in vivo can lead to effective T-cell activation and elicit host T-cell responses to tumor cells. Possible mechanisms for this may be the small size of scFv-NKG2D (~50 kD) and existence of flexible linkers which allow efficient formation of immunologic synapses and reduce a steric barrier imposed by large, abundant glycoproteins such as CD43 and CD45 (24). In adult humans, about one half of the T cells are memory cells based on the expression of a memory marker CD45RO and their ability to respond to allogeneic cells (25). Therefore, these memory T cells may be primed by NKG2D ligand—positive tumor cells in the presence of scFv-NKG2D. Isotype control, black line; anti-Rae1-APC, gray line.

Immunosuppressive cells within the tumor microenvironment have been shown to prevent antitumor immunity (28, 29). Effective therapies must not only attack the tumor but must also change the local microenvironment leading to...
inhibition of tumor growth. Recent studies have shown that myeloid suppressive cells and Tregs express NKG2D ligands (10, 30). Both cell types make significant contribution in the immunosuppression of tumor microenvironment. The findings that even NKG2D ligand–deficient tumor cells can be affected by scFv-NKG2D treatment suggest that scFv-NKG2D allows T cells to attack host immunosuppressive cells. Nausch and colleagues showed that adoptive transfer of NK cells reduced numbers of CD11b+F4/80+Gr1+ MDSC cells but not the percentages of CD11b+F4/80+Gr1+ cells in tumor-bearing mice, suggesting that these MDSCs were targeted in vivo by adoptively transferred NK cells (10). Barber and colleagues showed that NKG2D can be used as a targeting strategy against Tregs within the tumor microenvironment (11). Our results showed that in both MC-38 and B16F10 models, MDSCs and Tregs expressed the NKG2D ligand Rae1, which is consistent with previous findings. Local expression of scFv-NKG2D in tumors significantly reduced the percentage of these immunosuppressive cells, providing more convincing evidence that MDSCs and Tregs can be targeted by scFv-NKG2D. 

Because NKT cells also express CD3, it is possible that scFv-NKG2D may engage NKT cells to tumor cells, leading to antitumor responses. It has been shown that administration of α-galactosylceramide (αGalCer, an agonist of NKT cells), αGalCer-loaded DCs, or αGalCer-loaded soluble CD1d molecule (αGalCer/sCD1d) could induce significant IFN-γ–producing NKT responses and protect against the development of metastases with B16 melanoma after (34, 35). However, success of αGalCer treatment in humans has been limited. The partial reason may be that in humans, there exist anti-α-linked sugar natural antibodies that do not exist in the mouse (36, 37). Lung and liver contain abundant NKT cells, which may play an important role in keeping tumor metastasis in check (34, 35, 38). Expression of membrane-bound anti-CD3 scFv and CD86 on B16 melanoma cells has been shown to suppress tumor growth in vivo and protect mice from B16 rechallenge. In the subcutaneous B16 model, NKT cells have been shown to play a critical role in in vivo antitumor activity (39). Therefore, it is possible that scFv-NKG2D may also engage both NKT and tumor cells, resulting in vivo antitumor activity. 

T-cell infiltration into tumors (especially CD8+ T cells) is often correlated with improved survival (40–42). Recent studies have shown that tumor-specific CD4+ T cells could also differentiate in vivo into cytotoxic T cells and induce regression of established melanoma (43, 44). The results that local expression of scFv-NKG2D increased recruitment of T cells and reduced tumor growth are consistent with these findings. The possible mechanisms for enhanced T-cell infiltration after scFv-NKG2D treatment may be that scFv-NKG2D engages NKG2D ligand–positive cells in...
tumors and resident and/or newly arrived T cells, resulting in T-cell activation and production of IFN-γ leading to IFN-γ–inducible chemokines such as CXCL9 and CXCL10 (the ligands for CXCXR3), CCL2 (the ligand for CCR2), and CCL11 (the ligand for CCR2 and CCR5; ref. 44). These chemokines may attract more T cells to tumor sites and help eliminate tumors.

Regulation of NKG2D ligand expression can occur on multiple levels (transcriptional, posttranscriptional, and posttranslational levels; ref. 9). DNA pathways initiated by ATM (ataxia telangiectasia, mutated) or ATR (ATM- and Rad3-related) protein kinases have been shown to play important roles in upregulation of NKG2D ligands (45). Many anticancer drugs can also upregulate NKG2D ligands (46), which not only potentially enhances the host NK cell antitumor activity but also suggests possible synergistic effects of these drugs with scFv-NKG2D treatment.

In view of the wide expression of NKG2D ligands on many human cancers and Tregs and MDSCs, the strategy described here has a promising potential to treat many human cancers by direct and indirect mechanisms. The ability of scFv-NKG2D to induce long-term immunologic memory may be beneficial to cancer patients with minimal residual disease. Collectively, our findings suggest that scFv-NKG2D represents a promising alternative to elicit host immunity for effective immunotherapy against cancer.

**Disclosure of Potential Conflicts of Interest**

The authors declare no financial or commercial conflict of interest. Dartmouth College has applied for patent protection for the scFv/NKG2D technology described in this article.

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