 ORIGINAL ARTICLE

Treatment of multiple myeloma with adoptively transferred chimeric NKG2D receptor-expressing T cells

A Barber1, KR Mecban2 and CL Sentman1

Multiple myeloma causes approximately 10% of all hematologic malignancies. We have previously shown that human T cells expressing chimeric NKG2D receptors (chNKG2D) consisting of NKG2D fused to the CD3ζ cytoplasmic domain secrete proinflammatory cytokines and kill human myeloma cells. In this study, we show chNKG2D T cells are effective in a murine model of multiple myeloma. Mice with established 5T33MM–green fluorescent protein tumors were treated with one or two infusions of chNKG2D T cells. Compared with mice treated with T cells expressing wild type (wt)NKG2D receptors, a single dose of chNKG2D T cells increased survival, with half of the chNKG2D T-cell-treated mice surviving long term. Two infusions of chNKG2D T cells led to tumor-free survival in all mice. ChNKG2D T cells were located at sites of tumor growth, including the bone marrow and spleen after intravenous injection. There was an increase in activated host T cells and NK cells at tumor sites and in serum interferon-γ after chNKG2D T-cell injection. Surviving mice were able to resist a rechallenge with 5T33MM cells but not RMA lymphoma cells, indicating that the mice developed a protective, specific memory response. These data demonstrate that chNKG2D T cells may be an effective adoptive cellular therapy for multiple myeloma.

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INTRODUCTION

Multiple myeloma, a B-cell malignancy characterized by the proliferation of a plasma cell clone, causes approximately 20% of deaths because of hematologic malignancies in the United States. Although current therapies including autologous stem cell transplant demonstrate high complete response rates, the majority of patients relapse.1 As an alternative to standard treatments, immunotherapy may be able to specifically target tumor cells. Immunotherapeutic approaches used to treat myeloma include idiotype vaccination, dendritic cell therapies and granulocyte-macrophage colony-stimulating factor-secreting tumor cell vaccines.2-3 Adoptive transfer of activated myeloma patient T cells or cells that are engineered to express T-cell receptors specific for tumor-associated antigens is another promising therapeutic approach.4,5 A proportion of myeloma patients have circulating T cells that are specific for myeloma-associated antigens, such as Mucin-1, NY-ESO-1, WT-1 or idiotype proteins, and the presence of tumor-specific T cells is associated with increased survival.6-9 However, in patients with myeloma, the tumor cells have somehow escaped immune recognition. This could occur for many reasons, such as a lack of co-stimulation during T-cell activation, downregulation of major histocompatibility complex class I molecules, or the presence of suppressive factors, such as regulatory T cells or immunosuppressive cytokines. Treatments that target the tumor and activate the host immune system to target myeloma cells may be necessary for long-term benefit.

All human myeloma samples express ligands for the NKG2D receptor.10-13 Most healthy tissues do not express ligands for NKG2D, hence NKG2D ligands are potential tumor-specific targets.14,15 Human CD8+ T cells and NK cells are able to lyse myeloma cells and secrete proinflammatory cytokines after interactions with NKG2D ligands on myeloma cells.10,12,16-18 This indicates that targeting myeloma cells via the NKG2D receptor is a potential therapeutic opportunity. We have previously demonstrated that human T cells expressing chimeric NKG2D receptors (chNKG2D) consisting of NKG2D fused to the CD3ζ cytoplasmic domain lyse human myeloma cells.19 ChNKG2D T cells also secrete proinflammatory cytokines when cultured with primary myeloma bone marrow samples but not when cultured with healthy bone marrow cells or autologous peripheral blood mononuclear cells.19 In addition, treatment of tumor-bearing mice with chNKG2D T cells leads to long-term, tumor-free survival in murine models of ovarian cancer and lymphoma.20,21 This study determined the in vivo antitumor efficacy of chNKG2D T cells using a murine model of multiple myeloma and evaluated the induction of tumor-specific host immune responses.

RESULTS

ChNKG2D T cells lyse 5T33MM–GFP cells and produce IFNγ when cultured with murine myeloma cells

5T33MM is a myeloma cell line that formed spontaneously in an aging B6.Kalwrij mouse and was subsequently isolated and adapted to grow in cell culture.22,23 When injected intravenously (i.v.) into B6.Kalwrij mice, these tumor cells traffic to the bone marrow and spleen (but not the lymph nodes) and lead to a spike in serum immunoglobulin; thus, this model recapitulates features of human multiple myeloma in...
immunocompetent, syngeneic mice. This study investigated the potential of using chNKG2D T cells in vivo as a therapy for myeloma. To determine whether 5T33MM cells were potential targets of chNKG2D T cells, the expression of NKG2D ligands was measured. 5T33MM—green fluorescent protein (GFP) cells expressed cell surface NKG2D ligands as detected by staining with a soluble NKG2D receptor (Figure 1a). Reverse transcriptase-PCR for three murine NKG2D ligands was also performed, and 5T33MM—GFP cells expressed mRNA for Rae1 and Mult1 but not H-60 (Figure 1b). Rae-1 cell surface expression was also confirmed by staining with anti-Rae1 antibodies (data not shown). ChNKG2D T cells lysed 5T33MM—GFP cells, and this lysis was dependent on the NKG2D receptor because incubating the T cells with blocking anti-NKG2D antibodies before the assay abolished killing of the tumor cells (Figure 1c). ChNKG2D T cells also secreted significant amounts of interferon (IFN) (23730 ± 798 pg/ml) compared with wild type (wt)NKG2D T cells (6.4 ± 5 pg/ml) when cultured with 5T33MM—GFP cells (Figure 1d). These data show that 5T33MM—GFP cells expressed NKG2D ligands and were recognized by chNKG2D T cells. These results were similar to previous data showing that human chNKG2D T cells lyse human myeloma cell lines in an NKG2D-dependent manner, and secrete proinflammatory cytokines, including IFNγ, when cultured with human myeloma cell lines and bone marrow from patients with myeloma.

**Treatment with chNKG2D T cells leads to long-term survival of 5T33MM—GFP-bearing mice**

To test the in vivo therapeutic efficacy of chNKG2D T cells against an established myeloma, 5T33MM—GFP cells were injected into B6. Kalwrij mice, and mice were given wtNKG2D or chNKG2D T cells. To determine whether mice had established tumors at the time of T-cell treatment, the number of 5T33MM—GFP cells was measured in the spleen and bone marrow of tumor-bearing mice beginning 5 days after tumor cell injection. Tumors were detected in the spleen and bone marrow 5 days after tumor cell injection, and increased over time (Figure 2a). Mice were given a single dose of wtNKG2D or chNKG2D T cells 12 days after tumor cell injection. The survival of the mice was measured. Although wtNKG2D T-cell-treated mice had a median survival of 28 days, all of the chNKG2D T-cell-treated mice had an increased survival, with approximately 30% of the chNKG2D T-cell-treated mice surviving long term (5 out of 11 mice) (Figure 2b). A previous study using a murine model of lymphoma had shown that multiple treatments with chNKG2D T cells enhanced antitumor efficacy. To increase the efficacy of treatment, tumor-bearing mice were injected with two treatments of wtNKG2D or chNKG2D T cells 5 and 12 days after tumor cell injection. All wtNKG2D T-cell-treated mice were killed because of tumor growth while all of the chNKG2D T-cell-treated mice survived long term (Figure 2c). These data show that chNKG2D T-cell treatment of established myeloma increased survival, and multiple doses of chNKG2D T cells led to long-term survival in all mice.

Lymphodepletion of the tumor-bearing host via radiation or chemotherapy has been shown to increase the in vivo efficacy of adoptively transferred T cells. To test whether lymphodepletion may increase the therapeutic potential of chNKG2D T cells, mice-bearing 5T33MM—GFP tumor cells were injected with cyclophosphamide (180 μg g⁻¹) or Hank’s balanced salt solution 3 days before injection of wtNKG2D or chNKG2D T cells. This cyclophosphamide treatment regimen is similar to what has been previously been shown to be lymphodepleting. Pretreating mice with cyclophosphamide before chNKG2D T-cell injection did not increase the survival of the mice any more than treatment with chNKG2D T cells alone. Two out of five chNKG2D T-cell-treated mice survived long term compared with two out of six mice treated with cyclophosphamide and chNKG2D T cells, indicating that lymphodepletion did not increase chNKG2D T-cell antitumor efficacy (Figures 3a and b). To ensure that the treatment with cyclophosphamide was lymphodepleting, mice-bearing 5T33MM—GFP tumor cells were injected with cyclophosphamide (180 μg g⁻¹) or phosphate-buffered saline, and the number of leukocytes was measured in the spleen and bone marrow after 3 days. As shown in Figure 3c, injection of cyclophosphamide led to a significant decrease in the number of CD19⁺ B cells, CD8⁺ and CD4⁺ T cells, NK cells, and GR1⁺ myeloid cells in the spleen and bone marrow of mice with 5T33MM—GFP tumor, compared with mice treated with phosphate-buffered saline.

**ChNKG2D T cells traffic to the bone marrow after i.v. injection in tumor-bearing mice**

5T33MM—GFP tumor cells were detected in the spleen and bone marrow, but not lymph nodes, after i.v. injection (data not shown). The localization of chNKG2D T cells was studied to determine whether the transferred cells are found in tumor-rich organs after
i.v. injection. ChNKG2D T cells that expressed a congenic marker, Ly5.1, were injected i.v. into mice 12 days after 5T33MM–GFP cell injection, and the presence of Ly5.1+ cells in the bone marrow and spleen was determined. One day after T-cell injection, chNKG2D T cells were found both in the spleen (14.4×10^4 chNKG2D T cells ± 1.01×10^4 out of 9.31×10^7 total spleen cells) and in the

Figure 2 Treatment with chNKG2D T cells leads to long-term survival of 5T33MM–GFP-bearing mice. 5T33MM–GFP cells (3×10^5) were injected i.v. into B6.Kalwrij mice on day 0. (a) Mice were killed 5, 12, 16 or 20 days after 5T33MM–GFP cell injection and tumor burden was determined by calculating the number of 5T33MM–GFP cells in the spleen and bone marrow (n=3). Naive mice were also analyzed at each timepoint. Mice were treated i.v. with (b) a single treatment of 5×10^6 wtNKG2D (squares) or chNKG2D (diamonds) T cells after 12 days or (c) two doses of wtNKG2D T cells or chNKG2D T cells after 5 and 12 days. Survival of the mice was determined. Data are combined from two independent experiments. Significant differences are indicated (**P<0.001).
bone marrow (2×10^6 chNKG2D T cells ± 0.1×10^4 out of 1.24×10^7 total bone marrow cells), although at a lower percentage in the bone marrow (Figure 4). Nested PCR for the chNKG2D receptor was also used to detect chNKG2D T cells in the bone marrow and spleen, and 1 day after T-cell injection, both the bone marrow and spleen were positive for chNKG2D T cells. Seven days after T-cell injection, chNKG2D T cells were no longer detected in either tissue by flow cytometry. These data show that chNKG2D T cells were located in both the spleen and bone marrow, and that these T cells did not survive long-term in vivo.

ChNKG2D T-cell treatment increases the activation of the host immune system

Previous studies in mouse models of ovarian cancer and lymphoma have shown that treatment with chNKG2D T cells induced a host immune response against tumor antigens and resulted in the mice demonstrating a resistance to tumor rechallenge. Activation of host cells was determined after chNKG2D T-cell infusion. One day after chNKG2D T-cell injection, the absolute numbers of host NK cells, CD4^+ and CD8^+ T cells did not change. However, host NK cells, CD4^+ and CD8^+ T cells in the bone marrow and spleen expressed more CD69 compared with mice treated with wtNKG2D T cells, indicating that host immune cells were more activated after chNKG2D T-cell treatment (Figure 5). Therefore, not only do chNKG2D T cells have direct antitumor effects on 5T33MM–GFP cells, but chNKG2D T cells contribute to the activation of host immune cells, which may target other tumor antigens.

We have previously shown in murine models of lymphoma and ovarian cancer that one effector molecule required for chNKG2D T-cell antitumor efficacy is IFNγ. To determine whether IFNγ was increased in vivo after chNKG2D T-cell treatment in 5T33MM–GFP tumor-bearing mice, the amount of IFNγ in the serum of tumor-bearing mice was measured. Serum IFNγ was increased in tumor-bearing mice treated with chNKG2D T cells compared with wtNKG2D T-cell-treated mice (Figure 5c). The increase in serum IFNγ was sustained for at least 1 week after chNKG2D T-cell injection, although the chNKG2D T cells were no longer present in significant numbers. Together these data show that chNKG2D T-cell treatment led to an increase in the activation of the host immune cells and in systemic IFNγ levels, indicating that chNKG2D T-cell treatment induced a host immune response to the tumor.
Tumor-surviving mice develop protective host memory responses to 5T33MM tumor antigens

To test whether tumor-surviving mice developed protective memory responses to tumor antigens, spleen cells from naïve mice or chNKG2D T-cell-treated surviving mice from Figure 2b were cultured with media alone, 5T33MM cells or with tumor cells the mice had not previously rejected, RMA and RMA-Rael cells, both murine T-cell lymphomas. Spleen cells from tumor-surviving mice secreted significant amounts of IFN\(\gamma\) when cultured with 5T33MM cells, but did not secrete IFN\(\gamma\) when cultured with media alone or with tumor cells the mice had not previously encountered, even if these tumor cells expressed ligands for NKG2D (Figure 6a). Spleen cells from naïve mice did not secrete IFN\(\gamma\) in any of the culture conditions.

To determine which cells were producing IFN\(\gamma\), intracellular staining was performed. As shown in Figure 6b, both CD8\(^+\) and CD4\(^+\) T cells from tumor-surviving mice produced IFN\(\gamma\) when cultured with 5T33MM cells, but not when cultured in media alone or with RMA-Rael cells. This showed that chNKG2D T-cell-treated tumor-surviving mice developed host memory responses to 5T33MM tumor antigens.

To determine whether the host memory responses were protective against a specific tumor rechallenge, 5T33MM–luciferase cells were injected i.v. into tumor-surviving mice from Figure 2c, 80 days after original tumor injection. Tumor cells were also injected into naïve mice as a tumor growth control. All of the tumor-surviving mice rejected the tumor rechallenge with 5T33MM–luciferase cells, whereas 5T33MM–luciferase-bearing naïve mice had a median survival of 27 days (Figure 6c). RMA-Rael-luciferase cells, a tumor cell line these mice had not previously been exposed to, were injected i.v. into additional 5T33MM–GFP tumor-surviving mice. RMA-Rael-luciferase cells grew at a similar rate in tumor-surviving mice and naïve mice, indicating that the protective memory response was specific for 5T33MM tumor cell antigens.

DISCUSSION

These data show that treatment with chNKG2D T cells leads to long-term survival of mice-bearing established multiple myeloma. In addition to increasing the survival of the mice, chNKG2D T cells are located in the bone marrow and increase the activation of host immune cells at the tumor site. Tumor-surviving mice develop protective host immune responses that are specific to myeloma cell antigens because surviving mice were able to reject a tumor rechallenge with myeloma cells but were unable to reject lymphoma cells. These findings combined with previous data in other tumor models indicate that chNKG2D T-cell therapy can lead to epitope spreading and the induction of long-term protective immunity.

Several mouse models for myeloma have been developed, with the severe combined immunodeficient-hu model and the 5T murine models being two of the most frequently used models.

One benefit of using a severe combined immunodeficient-hu model is that this model uses primary human myeloma cells inoculated into severe combined immunodeficient mice.

However, engrafting human cells into mice requires the use of immunodeficient mice, therefore this model is not optimal for evaluating therapeutic approaches that induce activation of the host immune system. Additionally, potential xenogeneic responses of infused human effector cells (that is, human anti-mouse responses) may occur complicating data interpretation.

An ideal mouse model for immunotherapeutic studies uses syngeneic tumor cells in an immunocompetent host. The 5T33MM murine myeloma model fulfills these criteria as injection of 5T33MM cells into B6.Kalwrij hosts leads to myeloma formation with symptoms similar to human myeloma.

Use of the 5T33MM mouse model not only allowed for the investigation of chNKG2D T-cell efficacy against myeloma cells in vivo, but also for the study of the induction of host immune responses to tumor antigens that would not be possible if an immunodeficient mouse model had been used. In addition, the 5T33MM murine model elicits a similar immunosuppressive environment as the human disease, including an increase in regulatory T cells, myeloid-derived suppressor cells and transforming growth factor-\(\beta\). This model also allows the study of the efficacy of chNKG2D T cells in an immunosuppressive environment that T cells would likely encounter in patients.
The role of different immune cells in developing productive antitumor responses against myeloma remains unclear. There is evidence that both CD8\(^+\) T cells and NK cells are able to lyse myeloma cells and to reduce myeloma tumor burden after activation with antigen or cytokines.\(^{17,41,42}\) The presence of tumor antigen-specific T cells in myeloma patients correlates with improved prognosis, and patient CD8\(^+\) T cells can lyse myeloma cells after activation, with lysis being dependent on major histocompatibility complex class I and perforin expression.\(^{2,4,6,7,43}\) However, there is much evidence that myeloma patients are immunosuppressed, which is partially because of myeloma cell expression of FasL, interleukin-10, interleukin-6, vascular endothelial growth factor, Muc-1, and transforming growth factor-\(\beta\).\(^{44}\) Increased amounts of these molecules, along with an increased number of regulatory T cells, can inhibit immune responses in myeloma patients.\(^{45-47}\) Thus, therapies that are able to reduce the effect of immunosuppressive mechanisms and fully activate the host immune response to respond to the tumor cells may demonstrate improved outcomes. In this study, chNKG2D T-cell treatment not only lead to tumor-free survival, but also the activation of the host immune response, as seen by an increase in activated host NK cells and T cells at the tumor site, and an increase in IFNg in the serum of the mice. This host immune response was also protective against a tumor rechallenge. These data indicate that chNKG2D T-cell therapy is a novel way to activate the host immune system to respond to myeloma antigens and may decrease tumor relapse by developing protective memory responses.

Unlike other adoptive cell therapy approaches that utilize tumor-specific T cells, treatment with chNKG2D T cells was not enhanced by lymphodepletion of the host. Proposed benefits of depleting immune cells before T-cell transfer include (1) long-term survival of transferred T cells through decreased competition for antigen-presenting cells (APCs) and homeostatic cytokines, (2) decreasing immunosuppressive cells, such as myeloid-derived suppressor cells and regulatory T cells, which decrease the antitumor responses of transferred T cells, (3) induction of homeostatic proliferation and activation of host T cells and (4) expansion of immature dendritic cells that can present tumor antigens.\(^{25,32,48-51}\) Although lymphodepletion has been shown to increase the survival of transferred T cells, this study shows that although chNKG2D T cells do not survive long term, they instead induce long-lived antitumor immune responses in the host. It has been previously demonstrated that host immune cells are essential for antitumor efficacy of chNKG2D T cells.\(^{35}\) The presence of host leukocytes at the time of chNKG2D T-cell injection is likely important for the induction of host immune responses, thus lymphodepletion may inhibit the development of the host antitumor response that results from chNKG2D T-cell treatment. Additionally, previous data have shown that chNKG2D T cells are able to decrease the number of regulatory T cells at the tumor site and activate host myeloid cells so that the local myeloid cells switch from an immunosuppressive to an immunostimulatory phenotype.\(^{36}\) As treatment with chNKG2D T cells recapitulated many of the benefits of lymphodepletion, including reducing immunosuppression and activating host immune cells, lymphodepleting the host before chNKG2D T-cell treatment does not appear necessary or beneficial.

Current chemotherapies for myeloma are inadequate. Although treatment with these drugs or autologous transplant offers a high response rate, the majority of patients still relapse. NKG2D ligand expression is partially regulated by the induction of the Ataxia telangectasia, mutated (ATM) and ATM and Rad3-related (ATR) DNA damage pathway and by ubiquitination and subsequent proteasomal degradation, thus chemotherapeutic agents that induce DNA damage or inhibit the proteasome may increase NKG2D ligand expression on the surface of cells.\(^{52,53}\) Supporting this idea, many agents used to treat myeloma, including bortezomib, melphalan, and doxorubicin, increase NKG2D ligand expression on primary myeloma cells, while thalidomide and dexamethasone did not.\(^{13,18,24}\) It is possible that coupling treatment with chemotherapeutic agents that upregulate the expression of NKG2D ligands with chNKG2D T-cell therapy may further enhance the therapeutic efficacy of chNKG2D T cells. However, additional studies should be performed to determine whether these compounds also upregulate NKG2D ligands on normal cells, which would might result in recognition of healthy tissues by chNKG2D T cells.

Treatment with chNKG2D T cells is a novel approach for treating myeloma. Treatment with chNKG2D T cells increased survival in mice bearing an established 5T33MM–GFP tumor, and these mice also developed protective host immune responses to tumor antigens. Unlike other adoptive cell therapy approaches, this was not enhanced by lymphodepletion of the host. All long-term surviving mice appeared healthy in this study and in previous studies, even 300 days after chNKG2D T-cell injection, indicating that treatment of tumor-bearing mice with chNKG2D T cells did not cause significant side effects.\(^{23,24}\) The fact that chNKG2D T cells do not survive long term to reconstitute the host may be a beneficial attribute and will minimize potential unwanted side effects. These data suggest that chNKG2D T cells are a potentially useful and novel immunotherapeutic approach for multiple myeloma.

**MATERIALS AND METHODS**

**Mice**

C57Bl/6 Ly5.2+ mice were purchased from the National Cancer Institute (Frederick, MD, USA). C57Bl/Ka/birj mice were kindly provided by Dr Randolph Noelle (Dartmouth Medical School, Lebanon, NH, USA). Male and female mice were between 7 and 10 weeks 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.

**Treatment of mice with 5T33MM cells and genetically modified T cells**

Mouse spleen cells were isolated and transduced with chNKG2D or wtNKG2D genes as previously described.\(^{20,25}\) 5T33MM–GFP and 5T33MM–luciferase cells were grown in DuBecco’s modified Eagle’s medium supplemented with 10% heat-inactivated fetal bovine serum, 100 U ml–1 penicillin, 100 \(\mu\)g ml–1 streptomycin, 1 \(\mu\)mol pyruvate, 10 mmol Hepes, 0.1 mmol non-essential amino acids and 50 \(\mu\)m 2-mercaptoethanol. 5T33MM–GFP cells (3 \(\times\)105) were injected i.v. into C57Bl/Ka/birj mice. For survival experiments, two doses of 5 \(\times\)106 wtNKG2D or chNKG2D T cells were transferred 5 and 12 days after tumor cell injection or a single dose of 5 \(\times\)106 wtNKG2D or chNKG2D T cells was transferred 12 days after tumor cell injection. Mice were monitored daily and were killed when they showed signs of morbidity, such as dragging of hind legs, scruffiness or labored breathing. For tumor rechallenge experiments, 3 \(\times\)105 5T33MM–luciferase or 3 \(\times\)105 RMA–Rae1–luciferase cells were injected i.v. into naive mice or tumor-surviving mice (day+80) that had previously received two doses of chNKG2D T cells on days 5 and 12 and survival of the mice was measured. In some experiments, cyclophosphamide (180 \(\mu\)g g–1, Sigma, St Louis, MO, USA) was administered intraperitonially 3 days before T-cell injection.

**Cytokine production and cytotoxicity by chNKG2D T cells**

WtNKG2D or chNKG2D T cells (106) were cultured with 5T33MM–GFP cells (106) or in media alone. Cell-free conditioned media were collected after 24 h and assayed for IFNg using mouse Duosel ELISA kits (R&D Systems, Minneapolis, MN, USA). Lysis of 5T33MM–GFP cells was determined by \(^{51}\)Cr release assays, as previously described.\(^{20,25}\) To block NKG2D receptors, T cells were preincubated at 37 °C for 2 h with anti-NKG2D mAbs (clone CX5, 20 \(\mu\)g ml–1, sodium azide free) or isotype control mAbs before addition of target cells.
Reverse transcriptase-PCR and nested PCR
Total RNA from ST33MM–GFP cells was extracted and used with primers for amplification of mouse NK22 ligand mRNAs, as previously described.20 For nested PCR, total DNA was isolated from spleen cells and bone marrow cells using a lysis buffer (50 mM Tris-HCl, 100 mM NaCl, 0.1% Triton-X 100, 1 mg ml⁻¹ proteinase K) at 5×10⁶ cells per 30 μL. Cells were lysed at 55 °C overnight, and proteinase K was inactivated at 98 °C for 10 min. These samples were directly used as template for nested PCR. The primers for the pFBneo vector and chNK22G were used as previously described.24 PCR products were run on agarose gels and visualized by staining with SYBR Safe (Invitrogen, Carlsbad, CA, USA).

Flow cytometry
For fluorescence-activated cell sorting analysis of NK22G ligand expression, ST33MM–GFP cells were stained with a mouse NK22–human immunoglobulin G1 fusion protein (R&D Systems) and an APC-labeled goat anti-human immunoglobulin G secondary (Jackson ImmunoResearch, West Grove, PA, USA) or with APC-anti-Rael (186107, R&D Systems). For ST33MM–GFP tumor burden analysis, mice were killed 5, 12, 16 or 20 days after tumor cell injection and analyzed for percent and absolute number of GFP⁺ cells in the spleen and bone marrow. For analysis of T-cell localization and host cell activation, mice were killed 1 or 7 days after T-cell injection. Spleen and bone marrow cells were incubated with FcR block and mouse anti-CD3 (clone 145–2C11) or anti-CD19 (clone 6D5), and biotin-conjugated anti-CD69 (clone H1.2F3) with a secondary incubation with PE-Cy5.5-conjugated anti-CD45.1 (clone A20) or anti-NK1.1 (clone PK136), and biotin-conjugated isothiocyanate-conjugated anti-CD3 (clone 145–2C11) or anti-CD4 (clone 145–2C11) with fluorescein isothiocyanate-conjugated anti-CD8 (clone 145–2C11). For intracellular staining, 10⁵ T33 cells were fixed and permeabilized by adding 150 μl of 4% paraformaldehyde to the wells (Sigma) for 30 min on ice, then treated with 1% BSA blocking buffer (Invitrogen, Carlsbad, CA, USA) for 20 min. Cells were then incubated with FcR block and cell-surface stained with fluorescein isothiocyanate-conjugated anti-CD8 (clone 145–2C11), APC-conjugated anti-CD4 (clone H1.2F3), and biotin-conjugated anti-CD3 (clone 145–2C11) or anti-NK1.1 (clone PK136) with a secondary incubation with PE-Cy5.5-conjugated streptavidin. All isotype controls were obtained from eBioscience (San Diego, CA, USA). Cell fluorescence was monitored using a FACSCalibur cytometer (Becton Dickinson, San Jose, CA, USA) or an Accuri C6 cytometer (Accuri Cytometers Inc., Ann Arbor, MI, USA).

Cytokine production in secondary stimulation cultures and in serum
Spleen cells (2.5×10⁶) from naive or day 80 tumor-surviving mice were cultured with irradiated (120 Gy) ST33MM, RMA-Rael or RMA cells (2.5×10⁶) for 72 h. Cell-free conditioned media or serum samples taken directly from mice were assayed for IFNγ by ELISA using mouse DuoSet ELISA kits (R&D Systems). For intracellular staining, 10⁶ T33 cells were killed 3 days after cyclophosphamide treatment. Spleen and bone marrow cells were incubated with FcR block and mouse anti-CD3 (clone 145–2C11) or anti-CD19 (clone 6D5), APC-conjugated anti-CD8 (clone H1.2F3) and anti-CD45 (clone RBD-8C5), and biotin-conjugated anti-CD3 (clone 145–2C11) or anti-NK1.1 (clone PK136) with a secondary incubation with PE-Cy5.5-conjugated streptavidin. All isotype controls were obtained from eBioscience (San Diego, CA, USA). Cell fluorescence was monitored using a FACSCalibur cytometer (Becton Dickinson, San Jose, CA, USA) or an Accuri C6 cytometer (Accuri Cytometers Inc., Ann Arbor, MI, USA).

Statistical analysis
Differences between groups were analyzed using the Student’s t-test or analysis of variance, as appropriate. Values of P < 0.05 were considered significant. For survival studies, Kaplan–Meier survival curves were plotted and analyzed using the log-rank test and Prism software (GraphPad Software, San Diego, CA, USA).

CONFLICT OF INTEREST
The technology described in this paper is licensed by Celdara Medical, LLC. Dr Sentman and Celdara are developing the technology for clinical use. If they are successful, Dr Sentman will receive compensation. This arrangement is under compliance with the policies of Dartmouth College.

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