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Mouse Tumor Vasculature Expresses NKG2D Ligands and Can Be Targeted by Chimeric NKG2D-Modified T Cells

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Tumor angiogenesis plays an important role in the development of solid tumors, and targeting the tumor vasculature has emerged as a strategy to prevent growth and progression of solid tumors. In this study, we show that murine tumor vasculature expresses Rae1, a ligand for a stimulatory NK receptor NKG2D. By genetic modification of T cells with an NKG2D-based chimeric Ag receptor, referred to as chNKG2D in which the NKG2D receptor is fused to the signaling domain of CD3ζ-chain, T cells were capable of targeting tumor vasculature leading to reduced tumor angiogenesis and tumor growth. This occurred even in tumors where the tumor cells themselves did not express NKG2D ligands. H5V, an endothelial cell line, expresses Rae1 and was lysed by chNKG2D-bearing T cells in a perforin-dependent manner. In vitro capillary tube formation was inhibited by chNKG2D T cells through IFN-γ and cell–cell contact mechanisms. The in vivo antiangiogenesis effects mediated by chNKG2D-bearing T cells at the tumor site were dependent on IFN-γ and perforin. These results provide a novel mechanism for NKG2D-based targeting of solid tumors.

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Materials and Methods

Mice and cell lines

C57BL/6 (B6; wild-type [wt]) were purchased from National Cancer Institute (Frederick, MD). IFN-γ–deficient mice B6.129S7-Ifng<sup>−/−</sup> (IFN-γ<sup>−/−</sup>) and perforin-deficient mice B6-Pfra<sup>tm1Sdz</sup>/J (Pfp<sup>−/−</sup>) were obtained from The Jackson Laboratory (Bar Harbor, ME). Animals used in experiments were between 7 and 12 wk of age. All experiments were conducted according to protocols approved by Dartmouth College’s Institutional Animal Care and Use Committee. B6-derived endothelial cell line H5V was obtained from Dr. José R. Conejo-García (The Wistar Institute). Murine colon cancer MC-38 cells (H<sup>2</sup>-<sup>−</sup>) were obtained from Dr. Richard J. Barth (Dartmouth’s Geisel School of Medicine). Mouse melanoma cell line B16F10 has been described previously (9). B16F10 cells are wtNKG2D- or chNKG2D-modified T cells (2

Flow cytometry

For determination of Rae1 expression in tumor vasculature, established B16F10 and MC-38 tumors (~12 mm in diameter) were excised, digested using a mixture of DNase and collagenase, according to the previously described protocol (15), and stained with allophtocyanin-labeled anti-pan Rae1 (R&D Systems, Minneapolis, MN), PE-labeled anti-CD45, and FITC-labeled anti-CD31 Mabs (BioLegend). All samples were pre- incubated with FcR block Abs (anti-mouse CD16/CD32, clone 93; eBioscience) to reduce nonspecific staining. Cell fluorescence was monitored using an Accuri C6 cytometer (Ann Arbor, MI). Flow cytometry analysis was performed using either Accuri or FlowJo software (Ashland, OR).

In vitro drug treatment of H5V cells

Regulation of NKG2D ligand expression on H5V endothelial cells was examined in response to H<sub>2</sub>O<sub>2</sub>-induced oxidative stress. H5V cells (4 × 10<sup>5</sup>) were plated in six-well plates in 2 ml complete media for 18–20 h before H<sub>2</sub>O<sub>2</sub> treatment. Two hours before treatment, cells were given with fresh media. Either H<sub>2</sub>O<sub>2</sub> (0.3 mM) or PBS was added to culture for 48 h to mimic oxidative stress. For studies examining whether ATM (ataxia telangiectasia mutated) and ATR (ATM- and Rad3-related protein kinase–mediated pathways were involved, caffeine (1 mM; Sigma-Aldrich, St. Louis, MO), an ATM and ATR inhibitor, or KU-55933 (10 μM; Sigma-

Retroviral transduction of primary T cells

Retroviral transduction of murine primary T cells was performed using ecotropic retroviruses, as previously described (14, 16). After transduction and expansion, 80–90% of the T cells are CD8<sup>+</sup> T cells and the remaining cells are CD4<sup>+</sup> T cells. Because few murine CD4<sup>+</sup> T cells express DAP10, the expression of NKG2D on the CD4<sup>+</sup> T cells is low (14). The chNKG2D gene consists of the full-length NKG2D fused with the cytoplasmic portion of CD3ζ, whereas wtNKG2D is the full-length endogenous NKG2D gene. Both of these NKG2D proteins associate with DAP10 in the transmembrane, but only chNKG2D can induce a primary signal in T cells via the CD3ζ cytoplasmic domain. Thus, wtNKG2D T cells serve as control T cells for the transduction process, infusion of activated T cells, and bind to NKG2D ligands, but the wtNKG2D receptor only signals through DAP10 and does not induce a primary signal through CD3ζ. Genetically modified effector T cells (6–7 d after transduction) were harvested, washed, and resuspended in cold HBSS before injection.

Cytotoxicity assays

Cytotoxicity of T cells was determined by a lactate dehydrogenase release assay using the CytoTox 96 Non-Radioactive Cytotoxicity Assay kit (Promega, Madison, WI). Specific lysis was determined by the following equation:

\[
\% \text{ Specific lysis} = \frac{\text{Experimental} - \text{Spontaneous Target}}{\text{Target Maximum} - \text{Spontaneous Target}} \times 100
\]

Tumor inoculation and T cell treatment

B16F10 (5 × 10<sup>5</sup>) tumor cells were injected s.c. into the shaved right flank of B6 mice. For treatment with T cells, wtNKG2D- or chNKG2D-modified T cells (2 × 10<sup>5</sup>) were administered intratumorally into mice 7 and 9 d after tumor injection. Tumors were measured every 2 d using a caliper starting on day 7, and tumor areas were calculated. To quantify tumor-associated vessels, we separated the tumor-inoculated skin from the underlying tissues, and only the vessels directly supplying the tumor were counted.

In vitro tube formation assay

The in vitro tube formation assay by mouse endothelial H5V cells was determined using an in vitro angiogenesis assay kit (Millipore, Billerica, MA). In brief,
prechilled (4°C) 48-well tissue culture plates were coated with growth factor–reduced Matrigel (100 μl/well; Becton Dickinson, Bedford, MA) and were incubated at 37°C for 1 h to allow the Matrigel to solidify. In control wells, H5V cells (4 × 10⁴/well) were suspended in 300 μl complete DMEM and gently added to the Matrigel-coated wells. Conditional media (CM) from activated T cells were collected as supernatants from overnight culture of either wtNKG2D- or chNKG2D-modified T cells (10⁶) in anti-NKG2D mAb (4 μg/ml)-coated 24-well non-tissue culture-treated plates. To determine whether soluble factors from activated T cells affected H5V tumor formation, we added CM at different dilutions to the H5V cultures in a total volume of 300 μl. Similarly, T cells were mixed with H5V cells at ratios ranging from 0.1:1 to 1:1 before addition to Matrigel-coated wells to determine the effects of T cells on H5V cell tube formation. After 6 h, media were removed, and cells were fixed with cold PBS-buffered 2% paraformaldehyde. Images were captured under phase-contrast microscopy at 40× magnifications using a Dino-Eye eyepiece digital camera (Microscope.com, Roanoke, VA).

In vivo angiogenesis assay

Growth factor–reduced Matrigel (Becton Dickinson, Franklin Lakes, NJ) mixed with an equal volume of B16F10 cells in a final volume of 500 μl was injected s.c. into wt B6 mice. T cells (2 × 10⁶, either wtNKG2D- or chNKG2D-modified T cells in 100 μl HBSS) were inoculated into Matrigel plugs on days 5 and 7 postimplantation. Two days after the final T cell injection, Matrigel plugs were isolated and hemoglobin content determined using Drabkin’s reagent (DS941; Sigma-Aldrich) according to the manufacturer’s instruction. Experiments were done twice.

Isolation of tumor-derived endothelial cells and MDSCs

Established B16F10 and MC-38 tumors (~12 mm in diameter) were excised, digested using cocktails of DNase and collagenase, according to the previously described protocol (15). The liberated cells were filtered through 70-μm nylon mesh (BD Falcon, Bedford, MA), followed by a density gradient centrifugation over Histopaque-1077 (Sigma) to remove dead cells. CD45+ cells were depleted from the cell samples using magnetic cell separation (MACS) with anti-CD45 Abs and LS columns (Miltenyi Biotec). The negative fraction was collected and purified using CD31+ magnetic beads (Miltenyi Biotec). The purity of CD45+ CD31+ cells was >85%. Tumor-derived MDSCs were sorted using MACS columns with anti-F4/80 mAbs. The purity of F4/80+CD11b+ cells was >95%.

Cytokine production by T cells

To determine whether chNKG2D T cells responded to tumor-derived endothelial cells, we cocultured T cells (10⁶) with purified CD31+ cells (10⁴) in 96-well plates for 24 h. Cell-free supernatants were assayed for IFN-γ by ELISA using Duoset ELISA kits (R&D Systems).

Statistical analysis

Differences between groups were analyzed using a Student t test or ANOVA. The p values <0.05 were considered significant.

Results

Treatment with chimeric NKG2D T cells inhibits s.c. NKG2D ligand-negative B16F10 tumor growth and tumor-induced angiogenesis

To determine whether the treatment with chNKG2D T cells has any effects on tumor angiogenesis, we chose the mouse B16F10 melanoma model because this tumor cell does not express NKG2D ligands either in vitro or in vivo (9), so we could evaluate the nontumor-targeted mechanisms of chNKG2D T cells in vivo. T cell transfer was performed intratumorally (on days 7 and 9 after tumor inoculation) to minimize systemic effects of T cell infusion.
(Fig. 1A). As shown in Fig. 1B, intratumoral injection of chNKG2D T cells significantly inhibited B16F10 tumor growth (p < 0.01 from days 9–15). In addition, we observed a reduced number of vessels around the tumor mass in the chNKG2D T cell–treated group, as shown in Fig. 1C and ID. To further confirm the results, we performed an in vivo Matrigel assay. Intratumoral injection of chNKG2D T cells significantly reduced the hemoglobin content in the Matrigel compared with wtNKG2D T cells (Fig. 1E). Although the full-length NKG2D receptor transduced into wtNKG2D T cells can recognize ligands similarly to the chNKG2D receptor, NKG2D alone does not induce a primary activation signal or trigger effector functions in T cells. These results suggest that chNKG2D T cell treatment inhibited tumor-induced angiogenesis when given at the early stage of tumor growth.

**CD31+ endothelial cells isolated from B16F10 and MC-38 tumors express Rae-1 and can stimulate NKG2D CAR T cells to produce IFN-γ**

Because B16F10 cells do not express NKG2D ligands both in vivo and in vitro, the data indicated that tumor-associated cells, rather than tumor cells themselves, were targeted by NKG2D CAR T cells. Previous studies have shown that tumor-infiltrating cells, such as Tregs and MDSCs, can express NKG2D ligands in tumors (7, 8, 10). These findings prompted us to hypothesize that the tumor microenvironment may be involved in the induction of NKG2D ligands, and the tumor vasculature may also express NKG2D ligands. Therefore, we analyzed samples from well-established B16F10 tumors (diameters ~12 mm) for the expression of NKG2D ligands. Tumor-derived, but not normal tissue (heart endothelium)–derived, CD31+ cells expressed Rae-1, an NKG2D ligand (Fig. 2A, 2C). The result was confirmed in the mouse MC-38 colon cancer model. MC-38 tumor-derived CD31+ endothelial cells also expressed Rae1 (Fig. 2B). To determine whether chNKG2D T cells recognized these tumor vasculature cells, we purified CD31+ cells from either B16F10 or MC-38 tumors and cocultured them with chNKG2D T cells. As shown in Fig. 2D, chNKG2D T cells produced significant amounts of IFN-γ compared with wtNKG2D T cells when cocultured with tumor-derived endothelial cells. To assess Rae1 expression on other cells within these tumors, we determined Rae1 expression on infiltrating leukocytes (CD45+; see Supplemental Fig. 1). These data show that the leukocytes had no or very low expression, whereas the nonleukocytes had no expression of Rae1.

**The ATM/ATR pathway is involved in the upregulation of Rae1 on endothelial cells**

DNA damage by genotoxic stress can activate a pathway initiated by ATM and ATR, which has been shown to play a critical role in NKG2D ligand induction (17). Within tumor microenvironments, the ATM/ATR pathway is often activated because of hypoxia and reoxygenation in solid tumors (18–20). To determine whether this pathway was involved in the regulation of Rae1 expression on tumor-associated endothelial cells, a B6-derived endothelial cell line H5V was used. H5V cells are derived from the heart tissue of B6 mice and have been immortalized by expression of the large T Ag of the SV40 virus (21). As shown in Fig. 3A, H5V cells expressed Rae1. To mimic oxidative stress, we treated H5V cells with 0.3 mM H2O2 in the presence or absence of ATM/ATR pathway inhibitors, caffeine, and KU55933 for 48 h. As shown in Fig. 3A and 3B, H2O2 significantly increased Rae1 expression on H5V cells. Caffeine and KU55933 completely blocked H2O2-induced upregulation of Rae1 expression, suggesting that ATM/ATR pathway was regulating Rae1 expression on endothelial cells.

**FIGURE 3.** Oxidative stress induces the expression of Rae1 on mouse endothelial cells. (A) B6 mouse–derived endothelial cell line H5V cells were treated with 0.3 mM H2O2 or PBS for 48 h. Rae1 and CD31 cell-surface expression was assessed by flow cytometry. Shaded histograms represent isotype controls; solid-line histograms represent PBS-treated cells; dashed-line histograms represent H2O2-treated cells. Histograms are representative of at least three independent experiments. (B) H5V cells were incubated with 0.3 mM H2O2 in the presence of either 1 mM caffeine (an inhibitor of ATM and ATR) or 10 μM KU55933 (an ATM inhibitor) for 48 h. DMSO (0.02%) was used as a vehicle control. Surface expression of Rae1 and CD31 was analyzed by flow cytometry. The relative MFI values of Rae1 and CD31 expression were set as 100 in the vehicle control group. Results shown are pooled data from three independent experiments, and they are represented as mean ± SD. **p < 0.05 compared with DMSO controls or H2O2 plus ATM/ATR inhibitors. (C) chNKG2D T cells use perforin to kill Rae1+ H5V in vitro. Effector T cells derived from wt B6 or Pip−/− mice that were modified with either wtNKG2D (white bars) or chNKG2D (black bars) were cocultured with H5V cells at an E:T ratio of 5:1 in 5-h lactate dehydrogenase release assays. Data are presented as mean ± SD of triplicates and are representative from two independent experiments. **p < 0.01.
Both IFN-γ and perforin are important in NKG2D CAR T cell–mediated inhibition of in vitro angiogenesis

Next, we determined whether NKG2D CAR T cells recognized and responded to H5V cells. After overnight coculture of H5V cells with chNKG2D-modified T cells, significantly higher IFN-γ was produced as compared with coculture with wtNKG2D T cells. In addition, chNKG2D T cells efficiently lysed H5V cells (Fig. 3C). To determine whether perforin was involved in in vitro angiogenesis, we used T cells generated from perforin-deficient (Pfp<sup>−/−</sup>) mice. Pfp<sup>−/−</sup> chNKG2D T cells were unable to kill H5V cells, indicating its critical role in the killing process.

Similar to HUVECs, H5V cells can also form tubelike structures on extracellular matrix (Matrigel) and can be used to evaluate in vitro angiogenesis. Because chNKG2D T cells directly responded to H5V cells, we reasoned that in vitro tube formation by H5V cells may be disrupted by interaction with chNKG2D T cells. First, the effects of soluble factors from activated chNKG2D T cells were determined by mixing diluted cell–derived CM with H5V cells before plating on Matrigel. T cell CM was prepared from chNKG2D or wtNKG2D T cells after cross-linking with plate-bound anti-NKG2D mAbs. Compared with control media or wtNKG2D T cell–derived CM, the CM from activated chNKG2D T cells significantly inhibited H5V tube formation in a dose-dependent manner (Fig. 4A–C). Coculture of chNKG2D T cells and H5V cells at a low cell ratio (1:10) significantly reduces H5V tube formation (Fig. 4D). To understand the molecular mechanisms involved, we determined the effects of chNKG2D T cells to mediate antiangiogenesis using T cells derived from IFN-γ<sup>−/−</sup> or perforin-deficient (Pfp<sup>−/−</sup>) mice. As shown in Fig. 5, the CM from IFN-γ<sup>−/−</sup> chNKG2D T cells had a reduced ability to inhibit in vitro H5V tube formation. Furthermore, in vitro culture of chNKG2D T cells with H5V cells resulted in significant IFN-γ production, whereas coculture of wtNKG2D T cells with H5V cells produced little IFN-γ (Fig. 5D). Less disruption of H5V tube formation was also observed when Pfp<sup>−/−</sup> chNKG2D T cells were used. The in vitro angiogenesis assays indicated that both IFN-γ and perforin were involved in chNKG2D T cell–mediated inhibition of endothelial cell tube formation.

Inhibition of B16F10 tumor growth and angiogenesis by NKG2D CAR T cell treatment are IFN-γ and perforin dependent

IFN-γ has been shown to inhibit tumor angiogenesis (22). One mechanism of IFN-γ–mediated antiangiogenesis is induction of two potent antiangiogenesis CXC chemokines, CXCL9 and CXCL10 (23). In a colon cancer (MC-38) lung metastasis model, treatment of tumor-bearing mice with chNKG2D T cells led to elevation of IFN-γ in serum (32–470 pg/ml) and prolonged survival (Supplemental Fig. 2).

To determine the extent to which IFN-γ from chNKG2D T cells was involved in their in vivo antitumor effects, we used T cells derived from IFN-γ–deficient mice (IFN-γ<sup>−/−</sup>). As shown in Fig. 6, intratumoral administration of IFN-γ–deficient chNKG2D T cells into B16F10 tumors had an impaired ability to reduce tumor burden and angiogenesis as demonstrated by tumor growth and the numbers of blood vessels growing into tumors compared with chNKG2D T cells generated from wt mice, indicating the critical role for IFN-γ production by these transferred T cells in vivo. Thus, the production of cytokines was critical for the antitumor responses mediated by chNKG2D T cells in vivo. Considering the critical roles of perforin in chNKG2D T cell–mediated in vitro cytotoxicity against H5V cells, we tested whether perforin was also involved in chNKG2D T cell–mediated therapeutic efficacy in vivo. After intratumoral injection, Pfp<sup>−/−</sup> chNKG2D T cells resulted in a reduced capacity to reduce tumor burden and angiogenesis (Fig. 6A), indicating that perforin was also required for in vivo therapeutic effects.

Discussion

Angiogenesis is an important hallmark of solid tumors and plays a very important role in tumor development and metastasis (2).
Antiangiogenesis drugs, such as Avastin (anti-VEGF mAb), can significantly promote survival in some cancer patients (2). Besides Abs, small molecule inhibitors that target tyrosine kinase receptors have also been used for antiangiogenesis therapy. These tyrosine kinase targets include VEGF-R, fibroblast growth factor receptor, platelet-derived growth factor receptor, and Tie-2, which are known to play crucial roles in the angiogenesis in tumors (2, 24).

Adoptive cell therapy (ACT) with tumor Ag-specific T cells is a promising strategy to treat cancer, and evidence suggests that T cell immunity can be used to control tumor growth (25, 26). However, clinical efficacy of ACT is often negatively affected by an immunosuppressive tumor microenvironment (27–29). Aberrant tumor vasculature and limited expression of chemokines and adhesion molecules can lead to poor penetration of effector T cells into tumor stroma (27–30). In addition, effector functions of tumor-specific T cells can be impaired by immunosuppressive molecules, such as VEGF, TGF-β, and IL-10, and interaction with suppressor cells (i.e., MDSCs and Tregs) (31–33). Hypoxia-HIF1α-VEGF axis plays critical roles in creating and maintaining the immunosuppressive tumor microenvironment (1). Combination therapy of anti-VEGF mAbs with ACT increases T cell infiltration into tumors and can improve therapeutic efficacy (34).

Active immunization with a soluble VEGF-R2–pulsed dendritic...
chNKG2D T cell–derived IFN-γ and perforin are involved in chNKG2D T cell-mediated antitumor angiogenesis in vivo. (A) Tumor-bearing mice that were inoculated s.c. with $5 \times 10^5$ B16F10 cells on day 0 were treated with two doses of chNKG2D T cells ($2 \times 10^5$) derived from either wt B6 (♦), IFN-γ−/− (▴), or Pfp−/− (●) mice on days 7 and 9. wtNKG2D transduced T cells (●) from B6 mice were used as negative controls. Tumor diameters were measured every 2 d starting 7 d after tumor injection. Results shown are pooled data from two independent experiments. Tumor areas are represented as mean ± SEM. *$p < 0.05$, **$p < 0.01$. (B) Representative images of tumor-inoculated sites in T cell–treated mice on day 15. (C) Tumor-inoculated sites were isolated from T cell–treated mice at day 15 and tumor-supplying vessels were counted. Data are shown as the individual values of eight mice in each group, pooled from two independent experiments. *$p < 0.01$ compared with wtNKG2D T cells. †$p < 0.01$ compared with IFN-γ−/− or Pfp−/− chNKG2D T cells.

Expression of NKG2D ligands is not restricted to tumor cells. Tumor-associated immunosuppressors including MDSCs and Tregs can also express NKG2D ligands and be targeted by NKG2D-based strategies (8, 36). In addition, MDSCs and Tregs have been shown to promote tumor angiogenesis (37, 38). In vitro, MDSCs can be targeted by chNKG2D T cells, leading to reduced production of a proangiogenic factor VEGF (Supplemental Fig. 3). A new finding in this study is that tumor vessels express NKG2D ligands; therefore, it may be possible to target the tumor vasculature with NKG2D CAR T cells. Because NKG2D CAR T cells can recognize all cells that express NKG2D ligands (e.g., tumor cells, tumor endothelium, and immunosuppressive cells), it is likely that the CAR T cells are targeting all of these cells to some extent. Thus, NKG2D CAR T cells can directly inhibit tumor angiogenesis through recognition of ligands on tumor endothelium and indirectly through production of cytokines within the local microenvironment, and by attacking ligand-expressing tumor cells and immunosuppressive cells that promote angiogenesis.

DNA pathways initiated by ATM or ATR protein kinases have been shown to play important roles in upregulation of NKG2D ligands (17). Hammond et al. (19, 39) have shown that hypoxia and reperfusion in solid tumors activate the ATR/ATF pathway via inducing DNA damage. Our results demonstrate that ATM/ ATR pathway inhibitors KU55933 and caffeine inhibited H$_2$O$_2$–induced Rae1 upregulation, indicating a possible role of the ATM/ ATR pathway in NKG2D ligand expression on tumor blood vessels. In addition, Hamerman et al. (40) have shown that Rae1 expression on macrophages can be induced by LPS, suggesting that the NF-$\kappa$B pathway is involved in Rae1 regulation. It has been shown that the NF-$\kappa$B signaling pathway is important in cancer-related inflammation and malignant progression (41, 42). Therefore, it is possible that induction of Rae1 expression is related to the damage and inflammation within the tumor microenvironment.

NK cells have been shown to inhibit tumor angiogenesis, especially during IL-12 treatment (43, 44). In a xenograft mouse model, local treatment with IL-12 induced tumor necrosis, vascular damage, and NK cell infiltration surrounding small vessels.
in compliance with guidelines from Dartmouth College.

Disclosures

The chimeric NKG2D technology used in this study is licensed to Celdara

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