Generation of Antitumor Responses by Genetic Modification of Primary Human T Cells with a Chimeric NKG2D Receptor

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Abstract

To create more effective T cells against human tumors, we have designed a strategy to allow T cells to recognize tumor cells using natural killer (NK) cell receptors but retain the effector responses of T lymphocytes. NKG2D is an activating cell surface receptor expressed on NK cells and on some T-cell subsets. Its ligands are primarily expressed on tumor cells. We have shown that by linking mouse NKG2D to the CD3ζ chain, it was possible to generate a chimeric NKG2D (chNKG2D) receptor that allowed activation of murine T cells on engagement with NKG2D ligand-positive tumor cells leading to antitumor responses in mice. In this study, a human version of the chNKG2D receptor was expressed on primary human T cells, and antitumor responses were determined. Human peripheral blood mononuclear cell–derived T cells were retrovirally transduced with a human *chNKG2D* receptor gene. These chNKG2D-bearing human T cells responded to NKG2D ligand-positive tumor cells by producing T-helper 1 cytokines, proinflammatory chemokines, and significant cellular cytotoxicity. This response could be blocked by anti-NKG2D antibodies, and it was dependent on NKG2D ligand expression on the target cells but not on expression of MHC molecules. In addition, the activity of chNKG2D-bearing T cells remained unimpaired after exposure to a soluble NKG2D ligand, soluble MICA, at concentrations as high as 1.5 μg/mL. These data indicate the feasibility of using chNKG2D receptors in primary human T cells and suggest that this approach may be a promising means for cancer immunotherapy. (Cancer Res 2006; 66(11): 5927-33)

Introduction

As a part of innate immunity, natural killer (NK) cells play an important role in prevention of tumor growth. NK cells attack tumors in the absence of MHC restriction using a combination of signals from activating and inhibitory receptors (1, 2). Adoptive transfer of activated or allogeneic NK cells is effective in treatment of certain types of leukemia and solid tumors (3, 4). However, in many cases, NK cell–mediated antitumor responses are weak, which may be due to the expression of inhibitory receptors, poor capacity for survival, or limited migration of effector cells into tumor sites (3, 5, 6). In contrast, T cells can migrate efficiently into various tissues and proliferate well in response to antigen stimulation (4, 7). However, T cells have strict specificities dictated by antigen-specific T-cell receptors (TCR). Propagation of large numbers (>10^{10}) of tumor-specific T cells for adoptive transfer from a small percentage (usually <0.01%) of the whole T-cell repertoire is difficult (7, 8). By transducing T cells with modified NK cell–activating receptors, it may be possible to combine the broad specificity of NK cells with the efficient migration, expansion, and memory capacity of T cells. This combination of NK cell recognition and T-cell effector functions may lead to stronger antitumor responses.

NK cells can use multiple activating receptors to recognize target cells. However, the ligands for many of them remain poorly defined, and some are expressed on normal tissues (1, 2). For cancer immunotherapy, it would be ideal to use those activating NK receptors, where ligand expression is preferentially on tumor cells to minimize the risk of autoimmunity. In humans, NKG2D receptors are expressed on NK cells, NKT cells, γδ T cells, and $CD8^+$ αβ T cells (9). Ligands for human NKG2D include MICA, MICB, UL-16-binding proteins, and Letal (9, 10). NKG2D ligands are primarily expressed on tumor cells but are absent on most normal tissues. In a previous study, we described a novel strategy to redirect murine T cells against tumors through a chimeric NKG2D (chNKG2D) receptor (11). The chNKG2D receptor contains NKG2D fused to a CD3ζ cytoplasmic domain, which allows direct activation of T cells via the Syk family tyrosine kinase cascade after engagement with NKG2D ligand-positive tumor cells. Besides direct killing of NKG2D ligand-positive tumor cells, the chNKG2D-bearing T cells were able to induce host immunity against related tumors that did not express NKG2D ligands in mice (11).

In this study, we describe a version of the chNKG2D receptor for use in primary human T cells. Our results showed that chNKG2D-modified T cells produced large amounts of T-helper 1 cytokines and lysed target cells in a NKG2D ligand-dependent manner. chNKG2D-mediated responses require the presence of NKG2D ligands but not MHC expression on target cells. In addition, chNKG2D-bearing T cells were resistant to inhibition by high concentrations of soluble MICA (sMICA). These data support the possibility of immunotherapy of human cancers using chNKG2D-modified T cells.

Materials and Methods

Cell lines. Cell lines Bosc23, PT67, P815, K652 (human myeloid leukemia cell line), U937 (human promonocytic leukemia cell line), and Jurkat were obtained from the American Type Culture Collection (Rockville, MD). Breast cancer cell lines MCF-7 and T47D were provided by Dr. James Direnzo (Dartmouth Medical School, Lebanon, NH). Pancreatic cancer cell line Panc-1 was provided by Dr. Murray Korc (Dartmouth Medical School). Prostate cancer cell lines PC-3 and DU145 and melanoma cell line A375 were provided by Dr. Marc Ernstoff (Dartmouth Medical School). Packaging cells Bosc23 and PT67 were grown in DMEM with a high glucose concentration (4.5 g/L) supplemented with 10% heat-inactivated fetal bovine serum (Hyclone, Logan, UT), 100 units/mL penicillin, 100 μg/mL streptomycin, 1 mmol/L pyruvate, 10 mmol/L HEPES, 0.1 mmol/L

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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nonessential amino acids, and 50 μmol/L 2-mercaptoethanol. All other cell lines were cultured in RPMI plus the same supplements as in DMEM.

Retroviral vector construction. The full-length human NKG2D cDNA was purchased from Open Biosystems (Huntsville, AL). Human CD3ζ chain and Dap10 cDNAs were cloned by reverse transcription-PCR (RT-PCR) using RNAs from Jurkat and U937 cells, respectively. All PCRs were done using High-Fidelity DNA Polymerase PfuUltra (Stratagene, La Jolla, CA) or Phusion (New England Biolabs, Ipswich, MA). All oligos were synthesized by Integrated DNA Technologies (Coralville, IA). chNKG2D was created by fusing the human CD3ζ chain cytoplasmic region coding sequence (CD3ζ-CYP) to the full-length gene of human NKG2D. *chNKG2D*, wild-type NKG2D (*wtNKG2D*), human *Dap10*, and *MICA* genes were cloned into a retroviral vector pFB-Neo (Stratagene). In some cases, a modified vector pFB-IRES-GFP was used to allow coexpression of green fluorescent protein (GFP) with genes of interest (11). A soluble human *NKG2D* gene was made by fusion of the extracellular portion of human NKG2D (73-216) with the mouse IgG1 hinge-CH2-CH3 portion (243-469). To allow the fusion protein to be secreted, mouse Dap10 signal peptide (1–18) was added to the NH_2 terminal of the fusion protein. To make a sMICA plasmid, the DNA fragment coding for extracellular domain of MICA (1-308) was amplified with a histine tag (6 × His) at the COOH terminal. Both *NKG2D-mIgG1* and *sMICA* genes were cloned into pEGFP-C1 vector (BD Clontech, Palo Alto, CA) by replacing the *EGFP* gene.

Production of soluble human NKG2D-mIgG1 protein and sMICA. For production of soluble human NKG2D protein, Bosc23 cells were transiently transfected with the NKG2D-mIgG1 vector by Fugene 6 (Roche, Indianapolis, IN) according to the manufacturer's instructions. Cell culture was done in serum-free medium. Supernatants were collected every 24 hours starting from 2 days after transfection for an additional 5 days. Collected supernatants were then precipitated by 50% saturated ammonium sulfate and resuspended in 5% original volume of PBS. Crude NKG2D-mIgG1 protein [in 1.5 mol/L glycine and 3 mol/L NaCl (pH 8.5)] was subjected to affinity chromatography using a rProtein A FF column (Amersham Biosciences, Piscataway, NJ) according to the manufacturer's instruction. Eluted fractions were then concentrated and desalted using Amicon Ultra columns (50K MWCO, Millipore, Billerica, MA). Purified NKG2D-mIgG1 protein was adjusted in PBS, filtered (0.22 μm), and stored in 4°C in the presence of 0.1% sodium azide. Generation and purification of sMICA were done in a similar way as human NKG2D-mIgG1, except that the collected supernatants (10 mmol/L imidazole was added) were loaded onto HisTrap columns (Amersham Biosciences) according to the manufacturer's instruction. In addition, purified sMICA was kept sterile in 4°C in the absence of 0.1% sodium azide.

Retrovirus production and transduction. Dualtropic packaging cell line PT67 was used to generate retroviruses for transduction of human peripheral blood mononuclear cells (PBMC). Virus-producing PT67 cells were prepared by cross-infection using ecotropic viruses collected from retroviral plasmid-transfected Bosc23 cells as described previously (11, 12). Human PBMCs were isolated from healthy donors by density gradient centrifugation using lymphocyte separation medium (density, 1.077; Mediatech, Herndon, VA) according to the manufacturer's instructions. All procedures were approved by the Internal Review Board at Dartmouth College. PBMCs were then stimulated with phytohemagglutinin (1 μg/mL) for 3 days. Retroviral transduction of PBMCs was done in 12-well plates with collected retroviruses from PT67 in the presence of 50 or 100 IU human interleukin (IL)-2 and 8 μg/mL polybrene. Cells ($\sim 0.5 \times 10^6$/mL) were spun at 32°C at 1,500 × *g* for 60 minutes. Viral supernatants were changed to complete RPMI after 8 or 20 hours. Two days after infection, transduced primary T cells ($\sim 0.5 \times 10^6$/mL) were selected in RPMI-10 medium containing G418 (0.5 mg/mL) plus 50 or 100 units/mL recombinant human IL-2 for an additional 3 days. NKG2D ligand MICA-expressing P815 (P815/MICA) cells were established by triple transduction with PT67-derived MICA-expressing vectors followed by G418 (1 mg/mL) selection for 10 days. Both wtNKG2D- and chNKG2D-expressing B3Z cells (B3Z/wtNKG2D and B3Z/chNKG2D) were established using a similar protocol, except that cotransduction of the *NKG2D* genes with the mouse *Dap10* gene was done. NKG2D-positive B3Z cells were then isolated by cell sorting.

RT-PCR. Total RNAs from vector-, wtNKG2D-, and chNKG2D-transduced primary human T cells were extracted using the RNeasy Mini kit (Qiagen, Valencia, CA). Synthesis of first strands of cDNAs using random hexamer primers (Fermentas, Hanover, MD) was done according to the manufacturer's instructions. The resulting cDNA, corresponding to 33 ng of total RNA, was subjected to PCR amplification in a total volume of 20 μL buffer, including 0.5 μmol/L of each primer, 0.2 mmol/L of each deoxynucleotide triphosphate, and 1 unit Taq DNA polymerase (New England Biolabs). The primers used for amplification of the human β-actin sequence were 5′-GATCATTGCTCCTCCTGAGC-3′ (β-actin sense) and 5′-CGTCATACTC-CTGCTTGCTG-3′ (β-actin antisense). The primers used for amplification of the wtNKG2D sequence were 5′-CACAGCTGGGAGATGAGTGA-3′ (wtNKG2D sense) and 5′-TCGAGGCATAGAGTGCACAG-3′ (wtNKG2D antisense). The primers used for amplification of the chNKG2D sequence were 5′-AGGGCCAGAACCAGCTCTAT-3′ (chNKG2D sense) and 5′-AGA-AGGCTGGCATTTTGAGA-3′ (chNKG2D antisense). The PCR conditions were as follows: 95°C for 5 minutes followed by 30 cycles of 95°C for 30 seconds (denaturation), 57°C for 45 seconds (annealing), and 72°C for 1 minute (extension), with 5-minute incubation at 72°C at the end. The PCR products were run on agarose gels and visualized by staining with ethidium bromide.

Flow cytometry and magnetic cell sorting. For fluorescence-activated cell sorting analysis of NKG2D ligand expression, human tumor cells were stained with human NKG2D-mIgG1 fusion protein followed by staining with PE-labeled rat anti-mouse IgG1 (A85-1, BD PharMingen, San Diego, CA). MICA expression on P815 cells as determined by the human NKG2D/Fc chimera (R&D Systems, Minneapolis, MN) followed by staining with FITC-goat $F(ab')_2$ anti-human IgG (Caltag, Burlingame, CA). Purified anti-NKG2D antibody (1D11, mouse IgG1) was obtained from BD PharMingen. APC-anti-CD3 (S4.1) and PE-anti-NKG2D (1D11) were obtained from Caltag. FITC-anti-CD8 (RPA-T8), anti-CD4 (OKT4), and all isotype controls were obtained from eBiosciences (San Diego, CA). $CD8^+$ T cells were purified by a magnetic cell sorting kit (Miltenyi Biotec, Inc., Auburn, CA) according to the manufacturer's instruction and expanded for 2 more days without G418 before use as effector cells. Cell fluorescence was monitored using a FACSCalibur cytometer (Becton Dickinson, San Jose, CA). Cell sorting was done on a FACSAria sorter (Becton Dickinson).

Cytokine production by gene-modified T cells. For tumor cells grown in suspension, coculture with gene-modified primary human T cells (10^5) was done in round-bottom 96-well plates at a ratio of 1:1, whereas adherent tumor cells (2.5×10^4) were cocultured with T cells in flat-bottom plates. Tumor cells were irradiated (120 Gy) before use. Cell-free supernatants were collected after 24 and 72 hours. Twenty-four-hour supernatants were assayed for IFN-γ by ELISA using DuoSet ELISA kits (R&D Systems). Seventy-two-hour supernatants were used for detection of other cytokines using Bio-Plex kits (Bio-Rad, Hercules, CA) based on the manufacturer's protocol. Bio-Plex analysis was done by the Immune Monitoring Laboratory of the Norris Cotton Cancer Center (Lebanon, NH).

Cytotoxicity assay. Lysis of target cells was determined by a 4-hour ^{51}Cr release assay as described previously (11). To block NKG2D receptors, T cells were preincubated for an hour with the anti-NKG2D antibody (clone: 1D11, 20 μg/mL, sodium azide-free) before addition to the target cells. Inhibition experiments using sMICA (0-15 μg/mL) were done in a similar way except that the ratio of effector to target (E:T) was fixed at 10:1.

β-red staining. The LacZ activity in B3Z cells was determined by using a BetaRed β-galactosidase assay kit (EMD Biosciences, San Diego, CA) according to the manufacturer's instruction.

Statistical analysis. Differences between groups were analyzed using the Student's *t* test. *P*s < 0.05 were considered significant.

Results

Construction and expression of human chNKG2D receptors. The human chNKG2D receptor was made by fusing the cytoplasmic domain of human CD3ζ chain to the NH_2 terminal of the human NKG2D receptor. NKG2D is a type II protein, in which the NH_2 terminal is located intracellularly, whereas the CD3ζ

chain is a type I protein, with the COOH terminal in the cytoplasm (9, 11). On expression of chNKG2D, the orientation of the CD3ζ portion is reversed inside the cells. The extracellular and transmembrane domains are derived from NKG2D. The structures of the chNKG2D and wtNKG2D receptors used are diagramed in Fig. 1. To determine whether the *chNKG2D* receptor could be expressed in a similar manner as *wtNKG2D*, we cotransfected these genes with an adaptor protein gene (*Dap10*) into Bosc23 cells. wtNKG2D requires Dap10 to be expressed at the cell surface. A bicistronic vector with *GFP* gene downstream of internal ribosome entry site (IRES) was used to identify those cells that were transfected. NKG2D surface expression was analyzed by flow cytometry in the GFP^+ cell population. As expected, Bosc23 cells did not express either NKG2D or Dap10, and transfection with only one of the two components did not cause surface expression of NKG2D (Fig. 2*A*). However, cotransfection of a *NKG2D* gene along with an adaptor protein gene led to significant membrane expression of NKG2D. Surface expression of NKG2D was comparable after transfection with chNKG2D to those where the *wtNKG2D* gene was used. Thus, like wtNKG2D, human chNKG2D needs to be associated with an adaptor protein Dap10 for surface expression. chNKG2D and wtNKG2D expression in primary human T cells was initially determined by RT-PCR 7 days after retroviral transduction. As shown in Fig. 2*B*, wtNKG2D was expressed in vector-, wtNKG2D-, and chNKG2D-transduced human T cells, whereas chNKG2D expression was only found in chNKG2D-transduced T cells. The intensity of the wtNKG2D signal is greater in chNKG2D-transduced T cells because both wtNKG2D and chNKG2D can be templates for the wtNKG2D primers. This result indicated that chNKG2D could be expressed in primary T cells. Flow cytometry was then done on primary human T cells. Seven days after retroviral transduction, only a small percentage of $CD4^+$ T cells (data not shown) were positive for NKG2D, whereas majority of $CD8^+$ T cells expressed NKG2D (Fig. 2*C*). Compared with vector-transduced T cells, wtNKG2D and chNKG2D-modified $CD8^+$ T cells had higher NKG2D surface expression (>100-400% increase in geometric mean fluorescent intensity), supporting that the exogenously transduced *chNKG2D* gene can be expressed on the surface of T cells.

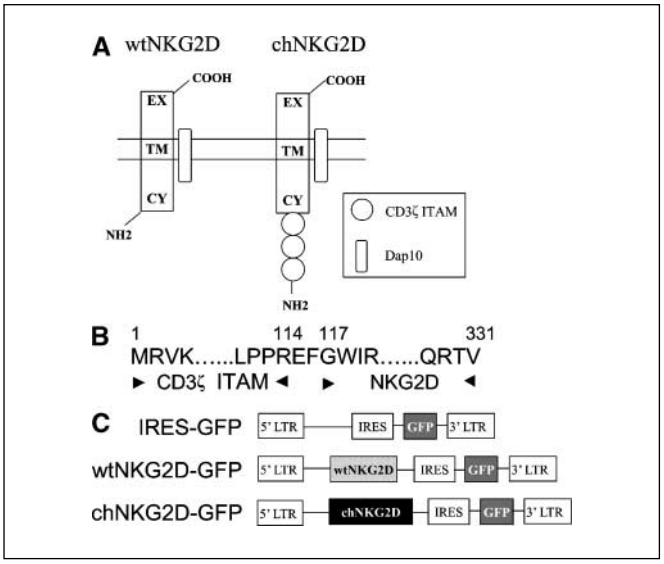

Figure 1. Structure of chNKG2D and retroviral constructs. *A,* schematic diagram of the wtNKG2D and chNKG2D receptors. Both receptors associate with Dap10 in the cell membrane, and the chimeric receptor has three CD3ζ immunoreceptor tyrosine-based activation motif (ITAM) regions added to the cytoplasmic (*CY*) domain of NKG2D. *EX,* extracellular domain; *TM,* transmembrane domain. *B,* amino acid sequence of the region where the three CD3ζ ITAMs is joined to the human NKG2D receptor. The three CD3ζ ITAM chains were fused to the NH_2 terminal of the NKG2D in a reverse orientation (COOH terminal to NH_2 terminal). *C,* NKG2D sequences were inserted into an empty IRES-GFP vector. The NKG2D was positioned behind the 5′-long terminal repeat (*5′ LTR*), and the GFP expression was controlled by the IRES.

Chimeric NKG2D-expressing T cells produce proinflammatory cytokines and chemokines on engagement with NKG2D ligand-positive tumor cells. A panel of human tumor cell lines was screened for NKG2D ligand expression using soluble human NKG2D-Ig fusion proteins. As shown in Supplementary Fig. S1, both hematopoietic and epithelial tumor cell lines are positive for NKG2D ligands. We determined whether the chNKG2D-transduced human T cells were able to recognize these NKG2D ligand-positive tumor cells. Because most $CD4^+$ T cells did not express NKG2D, we used purified $CD8^+$ T cells as effectors in all experiments. As shown in Table 1, chNKG2D-bearing T cells produced significant amounts of IFN-γ after coculture with NKG2D ligand-positive cells but not with ligand-negative P815 cells, indicating that these chNKG2D-modified T cells could functionally recognize NKG2D ligand-bearing tumor cells. In contrast, vector or wtNKG2D-modified T cells did not show any significant response to the stimulation by NKG2D ligand-positive cells. All T cells produce IFN-γ in the presence of RPMI8866 cells, although chNKG2D-bearing T cells produced 13-fold more IFN-γ than vector- or wtNKG2D-modified T cells. In addition, on chNKG2D engagement, chNKG2D-modified T cells also released significant amounts of proinflammatory chemokines [CCL3 (Fig. 3*A*) and CCL5 (Fig. 3*B*)] as well as other T-helper 1–type cytokines, granulocyte macrophage colony-stimulating factor (GM-CSF), and tumor necrosis factor-α (TNF-α; Fig. 3*C* and *D*), but not T-helper 2 cytokine IL-10 (Supplementary Fig. S2). These data indicate that a chNKG2D-NKG2D ligand interaction induces human T cells to produce T-helper 1–type cytokines.

chNKG2D-bearing human $CD8^+$ T cells lyse NKG2D ligand-positive tumor cells. The cytotoxic activity of chNKG2D-modified human $CD8^+$ T cells against various tumor cell lines was determined. As shown in Fig. 4*A*, chNKG2D-bearing T cells were able to lyse NKG2D ligand-positive target cells (P815/MICA, T47D, MCF-7, Panc-1, A375, K562, and RPMI8866) but not the ligand-negative cell line P815 *in vitro*. Similar to cytokine production, no significant killing was observed when either vector or wtNKG2D-modified T cells were used. In view of the fact that P815/MICA cells lack expression of human MHC class I and class II antigens and K562 cells are also negative for HLA class I antigens (13, 14), our data indicate that the chNKG2D-bearing T-cell–mediated killing of NKG2D ligand-positive tumor cells is MHC independent. To show the dependence of the cytotoxicity on NKG2D, we tested whether blocking antibodies to NKG2D reduced cytotoxic activity. As shown in Fig. 4*B*, chNKG2D-transduced T cells kill K562 and RPMI8866 cells, and this activity was reduced when anti-NKG2D antibodies were added. Vector-transduced T cells were unable to kill the target cells, and the activity was not changed with the addition of anti-NKG2D antibodies. These results showed that chNKG2D-bearing T cells kill ligand-positive tumor cells, and direct interaction between chNKG2D and NKG2D ligands is essential for chNKG2D-mediated functions.

Functional activity of chNKG2D-bearing T cells cannot be reduced by physiologic concentrations of sMICA. It has been reported that some cancer patients have elevated levels of sMICA, and NKG2D-mediated responses by NK and T cells were impaired by these cancer cell–derived soluble ligands (15, 16). To determine

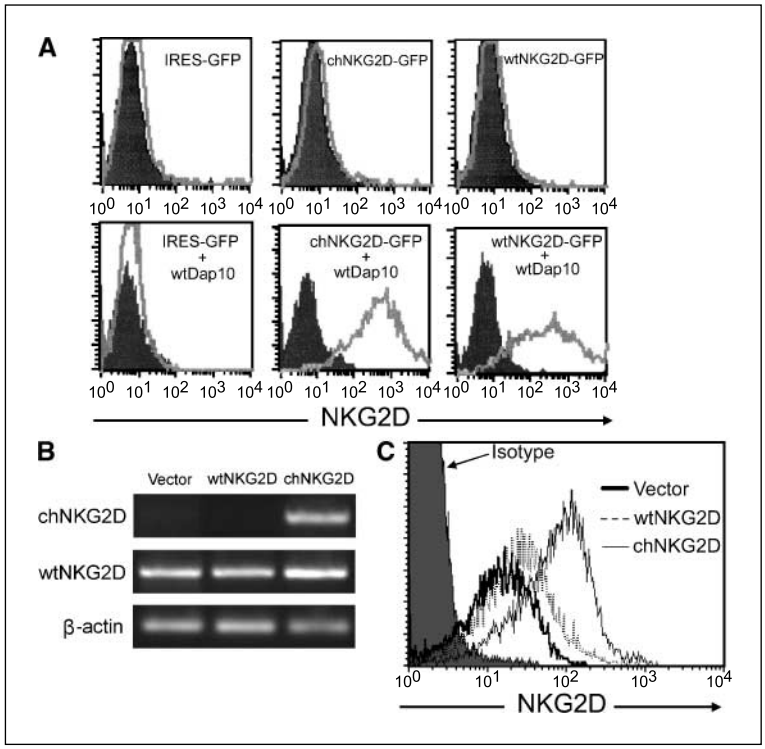

Figure 2. wtNKG2D and chNKG2D receptors are efficiently expressed on the cell surface. *A,* Bosc23 cells were transfected with plasmids containing *NKG2D-GFP* and *Dap10* adaptor genes. *Open histograms,* surface NKG2D expression was detected in the GFP^+ population using a PE-conjugated NKG2D antibody; *filled histograms,* isotype controls. *B,* primary human T cells transduced with virus-containing vector, wtNKG2D, or chNKG2D were analyzed by RT-PCR for gene expression using primers specific for chNKG2D, wtNKG2D, and β-actin. *C,* genetically modified human T cells were stained with PE-anti-NKG2D and FITC-anti-CD4 monoclonal antibodies. *Histograms,* cells were gated on $CD4^-$ ($CD8^+$) T lymphocytes.

whether soluble NKG2D ligands can modulate the functional activity of chNKG2D-bearing T cells, a recombinant sMICA protein that contains the extracellular domain of the MICA was produced. As shown in Fig. 5*A*, the expressed sMICA was ~55 kDa in a SDS-PAGE gel, consistent with previous findings (17). To confirm that the recombinant sMICA was functional, we tested whether plate-bound sMICA could activate B3Z cells that expressed chNKG2D. 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 immobilized sMICA to stimulate chNKG2D-expressing B3Z cells was evaluated by measurement of the $A_{570\ nm}$ values after addition of a β-red substrate. As shown in Fig. 5*B*, incubation of chNKG2D-bearing B3Z cells, but not wtNKG2D-bearing B3Z cells with immobilized sMICA, led to cell activation in a dose-dependent manner, indicating that the recombinant sMICA is functional. Next, we determined the extent to which the sMICA affects the activity of chNKG2D-bearing primary human T cells. There was no significant drop in the cytotoxicity of chNKG2D-bearing T cells against K562 and RPMI8866 cells when sMICA was added at a concentration of as high as 1.5 μg/mL (Fig. 5*C* and *D*). Reduced cytotoxicity (44.2%) against RPMI8866 was observed after addition of a high concentration of sMICA (15 μg/mL). However, the cytotoxicity against K562 remained unimpaired in the presence of sMICA at this concentration. These sMICA concentrations are much higher than those reported in sera from cancer patients (0.2-10 ng/mL). These data suggest that sMICA in cancer patients may not inhibit the function of chNKG2D-modified human T cells *in vivo*.

Discussion

Genetic modification of T cells with immune receptors, which are specific for tumor antigens or tumor cell–related surface proteins, provides a novel means for T cells to target tumor cells (18, 19). Gene transfer of TCR α and β chains has been shown to have an effect in T-cell–mediated killing of tumor cells both *in vitro* and *in vivo* (18–20). Down-regulation of HLA molecules on tumors, which is a common way for tumor cells to evade T-cell recognition, will render this strategy less effective (18). To overcome the dependence on the "MHC restriction" by TCR, chimeric antigen receptors (CAR) are designed to recognize intact membrane proteins. Most of CARs are "T bodies," which consist of a single-chain antibody (extracellular domain) that can bind a membrane

Table 1. IFN-γ production by chNKG2D-modified human T cells

	IFN-γ (pg/mL)			
Tumor cells	Vector	wtNKG2D	chNKG2D	No T cells
—	ND	ND	ND	—
P815	ND	ND	ND	ND
P815/MICA	ND	ND	230 ± 19	ND
RPMI8866	1,062 ± 84*	393 ± 27	13,942 ± 1,543	ND
K562	111 ± 14	ND	3,964 ± 347	ND
Jurkat	ND	ND	217 ± 23	ND
T47D	ND	ND	252 ± 8	ND
MCF-7	ND	ND	987 ± 181	ND
Panc-1	ND	ND	1,906 ± 122	ND
DU145	ND	ND	1,766 ± 122	ND
A375	ND	ND	520 ± 39	ND
PC-3	ND	ND	1,504 ± 31	ND

Abbreviation: ND, not detectable (<37 pg/mL).
*A representative result (mean ± SD of triplicates) of two or three independent experiments.

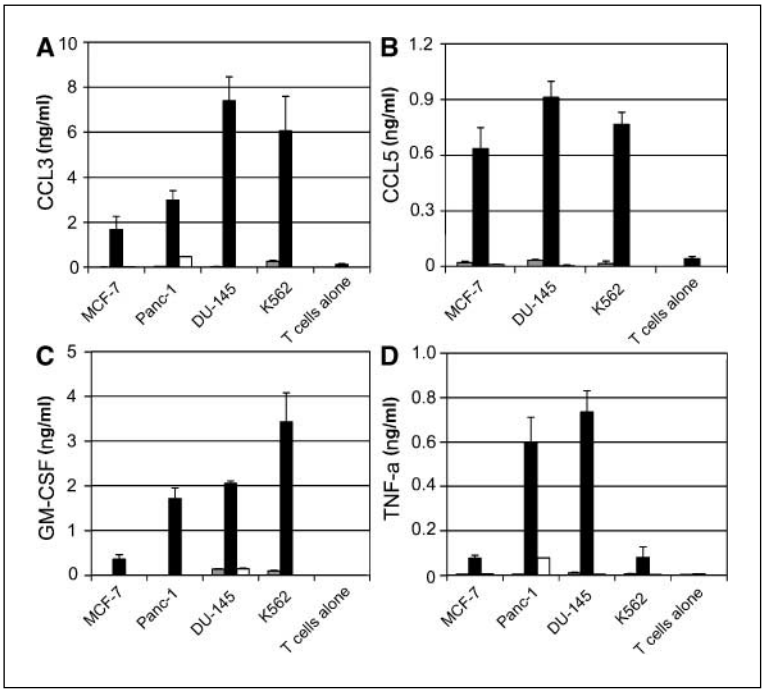

Figure 3. Chimeric NKG2D expressing T-cells secrete proinflammatory cytokines after coculture with ligand-expressing tumor cells. Human T cells transduced with wtNKG2D (*gray columns*) or chNKG2D (*black columns*) were cocultured with irradiated tumor cells for 72 hours. *White columns,* tumor cells. Culture supernatants were analyzed for the levels of chemokines (*A*) CCL3 or (*B*) CCL5 and cytokines (*C*) GM-CSF and (*D*) TNF-α by luminex. *Columns,* mean of two experiments done in triplicate; *bars,* SD.

antigen on the tumor cells, fused to a cytoplasmic signaling domain, such as the CD3ζ chain and Fc receptor γ chain (18–21). However, for a given tumor antigen, either high-affinity HLA-restricted TCRs or T bodies have to be identified and tested, which is not an easy task in many cases. In addition, the potential immunogenicity of the extracellular domain of T bodies may pose a problem for repeated infusion of T body–modified T cells (18–20).

Here, we reported that chNKG2D-modified human T cells were able to respond to NKG2D ligand-positive tumor cells *in vitro*, showing this as a possibility for immunotherapy of human cancers. One of the major advantages of this strategy is that chNKG2D-modified T cells can be used for immunotherapy of multiple types of malignant tumors, as long as tumors express NKG2D ligands. In this sense, chNKG2D receptor is a useful addition to the "CAR family". Many human cancers, such as colon cancer, leukemia, lymphoma, myeloma, cervical cancer, ovarian cancer, prostate cancer, and melanoma, have been found to express NKG2D ligands (9, 22–24). The ability of chNKG2D-bearing T cells to kill the pancreatic tumor cell line Panc-1 is especially interesting. Pancreatic cancer is the fourth leading cause of cancer death in the United States, accounting for ~30,000 death yearly (25). Therefore, infusion of chNKG2D-bearing T cells may be a potential

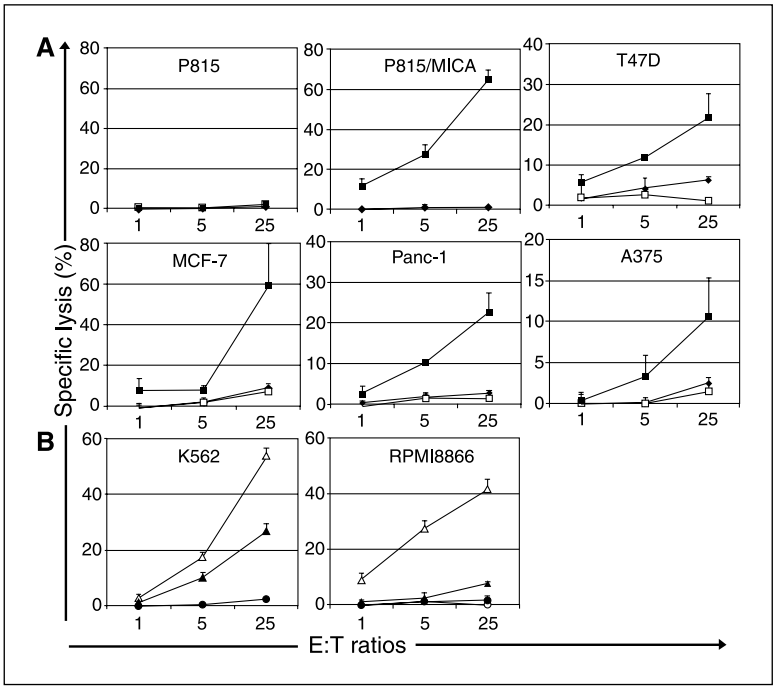

Figure 4. Specific lysis of NKG2D ligand-positive tumor cells by chNKG2D modified human CD8$^+$ T cells. *A,* primary human T cells transduced with empty vector (□), wtNKG2D (♦), or chNKG2D (■) were used as effector cells in a ^{51}Cr release assay. The ratios of effector to targets (E:T ratios) were 25:1, 5:1, and 1:1. chNKG2D-transduced T cells showed significantly higher ($P < 0.05$) cytotoxicity against NKG2D ligand-positive tumors (P815/MICA, T47D, MCF-7, Panc-1, and A375) but not ligand-negative P815 cells than vector- or wtNKG2D-transduced T cells at all ratios. *B,* for NKG2D blocking experiments, wtNKG2D-bearing (*circle*) or chNKG2D-bearing (*triangle*) CD8$^+$ T cells were incubated with saturating amounts of anti-NKG2D (1D11, *filled symbols*) or with control Ig (*open symbols*) before exposure to tumor cells. Blocking antibody 1D11 significantly reduced the cytotoxicity of chNKG2D-transduced T cells against K562 and RPMI8866 cells ($P < 0.05$) at all ratios compared with control antibody. *Points,* mean of two to three experiments done in triplicate; *bars,* SD.

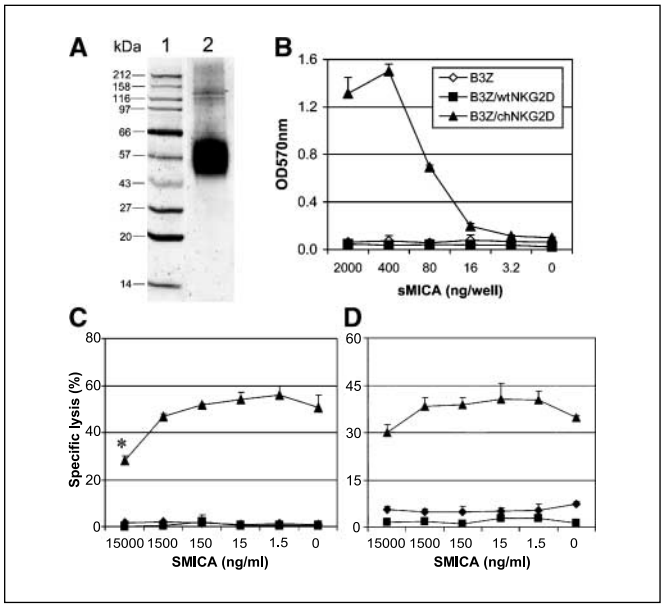

Figure 5. sMICA does not inhibit specific lysis of ligand expressing tumor cells at physiologic concentrations. *A, lane 2,* purified sMICA in the SDS-PAGE gel. *Lane 1,* molecular weight markers. *B,* B3Z, B3Z/wtNKG2D, and B3Z/chNKG2D cells were cultured in sMICA-coated plates, and the response to the ligand was measured by $A_{570\ nm}$ (*C* and *D*). Human primary T cells transduced with empty vector (♦), wtNKG2D (■), or chNKG2D (▲) were preincubated with varying concentrations of sMICA and then tested in a ^{51}Cr assay with RPMI8866 (*C*) or K562 (*D*) at an E:T ratio of 10:1. *Points,* mean of two experiments done in triplicate; *bars,* SD. *, $P < 0.05$, significant difference between the treated samples (in the presence of 15,000 ng/mL sMICA) and the untreated control.

treatment modality for this type of cancer. Unlike T bodies, chNKG2D has the same extracellular domain as wtNKG2D; thus, it would not be expected to induce an immune response against it.

After engagement with NKG2D ligand-positive tumor cells, chNKG2D-bearing human T cells produced significant amounts of IFN-γ, GM-CSF, and TNF-α, which were similar to what has been observed from activated human tumor-specific CTLs (26, 27). IFN-γ has been shown to activate antigen-presenting cells (such as dendritic cells and macrophages) to promote antigen presentation to CTLs (28). GM-CSF alone or in combination with TNF-α plays important roles in generation of mature dendritic cells as well as in tumor antigen presentation (29, 30). Phase I clinical trials using GM-CSF-modified tumor vaccines showed therapeutic effects in some cancer patients (31, 32). Therefore, interaction between chNKG2D-bearing T cells and NKG2D ligand-positive tumor cells may not only allow these T cells to kill tumor cells but also promote tumor antigen presentation due to local production of GM-CSF and IFN-γ. We observed differences in the amounts of cytokines produced when chimeric receptor-bearing T cells were cultured with different tumor cells. This may be due to the expression of different ligands for NKG2D on the tumor cell lines or different amounts of cytokine receptors expressed on the various tumor cell lines. Production of proinflammatory chemokines CCL3 (MIP-1α) and CCL5 (RANTES) has also been shown beneficial to antitumor immunity (33, 34). Taken together, production of T-helper 1 cytokines and proinflammatory chemokines by chNKG2D-bearing T cells is likely to promote host antitumor immunity.

The ability to generate functional activity by engagement of chNKG2D receptor suggests that this receptor leads to full activation of T cells, which is predicted due to the presence of CD3ζ signaling domain. Some studies have shown that inclusion of CD28 signaling domain into CARs could enhance the signal initiated by CD3ζ because CD28 provides a costimulation signal (18, 19). Similar to CD28, Dap10 transduces a costimulatory signal via a phosphatidylinositol 3-kinase pathway (9). In T cells, NKG2D associates with Dap10. Therefore, engagement of chNKG2D may activate T cells using both a primary signal through CD3ζ and a costimulatory signal through Dap10. However, further studies will be needed to clarify the details of chNKG2D-mediated signaling pathways.

It has been shown that human tumor cells produce soluble forms of NKG2D ligands and may lead to evasion from NK and T-cell surveillance (16, 17, 35); therefore, it was important to determine whether soluble ligands altered the function of chNKG2D-bearing T cells. Using a cytotoxicity assay, we have shown that chNKG2D-modified T cells remained functionally intact in the presence of sMICA at a level >1.5 μg/mL, which is a higher level (150- to 7,500-fold more) than those that found in sera of cancer patients (15, 36). However, it is possible that the levels of soluble NKG2D ligands at local tumor areas are different from that in sera. The mechanism for sMICA inhibition of cytotoxicity may be blocking of chNKG2D receptors or down-modulation of receptor expression. The cytotoxicity against RPMI8866 cells is more sensitive to sMICA as well as anti-NKG2D blocking antibody than that against K562 cells. The reason may be that RPMI8866 cells express lower level of NKG2D ligands.

There are two major safety concerns related to the use of chNKG2D. First, genetic modification of T cells may cause transformation of T cells due to preferable integration near transcriptional units, although the occurrence is rare (18, 19, 37). Use of nonviral vectors, such as transposon-based integration vector, may reduce the chance of transformation (38). Second, the risk of autoimmunity following infusion of autologous tumor-reactive T cells exists because NKG2D ligands have been found to be expressed by some normal tissues besides tumors, such as gut epithelial cells (9). One promising strategy to control the potential transformation and autoimmunity elicited by transferred T cells is coexpression of a "suicidal gene" along with the *chNKG2D* gene. Several suicidal genes, including a thymidine kinase (*TK*) gene from human herpes simplex virus and a Fas-based "artificial suicide gene," have been evaluated in terms of the ability to eliminate T cells (18, 19). A recent study showed that a modified human caspase-9-based molecular switch in combination with a small-molecule pharmaceutical drug (at concentration of 10 nmol/L) could induce apoptosis in 99% of transduced T cells both *in vitro* and *in vivo* (39). Engineering a suitable "suicide gene" into the vector along with the *chNKG2D* gene will enable the elimination of transferred T cells to prevent potential autoimmunity or T-cell transformation.

In summary, the present study suggests a new strategy for cancer immunotherapy using chNKG2D-modified human T cells, which can kill multiple types of human cancer cells (NKG2D ligand-positive) and produce T-helper 1 cytokines and proinflammatory chemokines simultaneously *in vitro*.

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References

1. Lanier LL. NK cell recognition. Annu Rev Immunol 2005;23:225–74.
2. Raulet DH. Interplay of natural killer cells and their receptors with the adaptive immune response. Nat Immunol 2004;5:996–1002.
3. Ruggeri L, Mancusi A, Capanni M, Martelli MF, Velardi A. Exploitation of alloreactive NK cells in adoptive immunotherapy of cancer. Curr Opin Immunol 2005;17:211–7.
4. Yannelli JR, Wroblewski JM. On the road to a tumor cell vaccine: 20 years of cellular immunotherapy. Vaccine 2004;23:97–113.
5. Felgar RE, Hiserodt JC. *In vivo* migration and tissue localization of highly purified lymphokine-activated killer cells (A-LAK cells) in tumor-bearing rats. Cell Immunol 1990;129:288–98.
6. Brand JM, Meller B, Von Hof K, et al. Kinetics and organ distribution of allogeneic natural killer lymphocytes transfused into patients suffering from renal cell carcinoma. Stem Cells Dev 2004;13:307–14.
7. Riddell SR. Finding a place for tumor-specific T cells in targeted cancer therapy. J Exp Med 2004;200:1533–7.
8. Ho WY, Yee C, Greenberg PD. Adoptive therapy with CD8(+) T cells: it may get by with a little help from its friends. J Clin Invest 2002;110:1415–7.
9. Raulet DH. Roles of the NKG2D immunoreceptor and its ligands. Nat Rev Immunol 2003;3:781–90.
10. Conejo-Garcia JR, Benencia F, Courreges MC, et al. Ovarian carcinoma expresses the NKG2D ligand Letal and promotes the survival and expansion of CD28$^-$ antitumor T cells. Cancer Res 2004;64:2175–82.
11. Zhang T, Lemoi BA, Sentman CL. Chimeric NK-receptor-bearing T cells mediate antitumor immunotherapy. Blood 2005;106:1544–51.
12. Zhang T, He X, Tsang TC, Harris DT. SING: a novel strategy for identifying tumor-specific, CTL-recognized tumor antigens. FASEB J 2004;18:600–2.
13. Nishimura M, Mitsunaga S, Akaza T, Mitomi Y, Tadokoro K, Juji T. Protection against natural killer cells by interferon-γ treatment of K562 cells cannot be explained by augmented major histocompatibility complex class I expression. Immunology 1994;83:75–80.
14. Day NE, Ugai H, Yokoyama KK, Ichiki AT. K-562 cells lack MHC class II expression due to an alternatively spliced CIITA transcript with a truncated coding region. Leuk Res 2003;27:1027–38.
15. Holdenrieder S, Stieber P, Peterfi A, Nagel D, Steinle A, Salih HR. Soluble MICA in malignant diseases. Int J Cancer 2006;118:684–7.
16. Groh V, Wu J, Yee C, Spies T. Tumour-derived soluble MIC ligands impair expression of NKG2D and T-cell activation. Nature 2002;419:734–8.
17. Salih HR, Rammensee HG, Steinle A. Cutting edge: down-regulation of MICA on human tumors by proteolytic shedding. J Immunol 2002;169:4098–102.
18. Sadelain M, Riviere I, Brentjens R. Targeting tumours with genetically enhanced T lymphocytes. Nat Rev Cancer 2003;3:35–45.
19. Rossig C, Brenner MK. Genetic modification of T lymphocytes for adoptive immunotherapy. Mol Ther 2004;10:5–18.
20. Ho WY, Blattman JN, Dossett ML, Yee C, Greenberg PD. Adoptive immunotherapy: engineering T cell responses as biologic weapons for tumor mass destruction. Cancer Cell 2003;3:431–7.
21. Hwu P, Shafer GE, Treisman J, et al. Lysis of ovarian cancer cells by human lymphocytes redirected with a chimeric gene composed of an antibody variable region and the Fc receptor γ chain. J Exp Med 1993;178:361–6.
22. Pende D, Rivera P, Marcenaro S, et al. Major histocompatibility complex class I-related chain A and UL16-binding protein expression on tumor cell lines of different histotypes: analysis of tumor susceptibility to NKG2D-dependent natural killer cell cytotoxicity. Cancer Res 2002;62:6178–86.
23. Groh V, Rhinehart R, Secrist H, et al. Broad tumor-associated expression and recognition by tumor-derived γ δ T cells of MICA and MICB. Proc Natl Acad Sci U S A 1999;96:6879–84.
24. Carbone E, Neri P, Mesuraca M, et al. HLA class I, NKG2D, and natural cytotoxicity receptors regulate multiple myeloma cell recognition by natural killer cells. Blood 2005;105:251–8.
25. Li D, Xie K, Wolff R, Abbruzzese JL. Pancreatic cancer. Lancet 2004;363:1049–57.
26. Rivoltini L, Kawakami Y, Sakaguchi K, et al. Induction of tumor-reactive CTL from peripheral blood and tumor-infiltrating lymphocytes of melanoma patients by *in vitro* stimulation with an immunodominant peptide of the human melanoma antigen MART-1. J Immunol 1995;154:2257–65.
27. Schwartzentruber DJ, Topalian SL, Mancini M, Rosenberg SA. Specific release of granulocyte-macrophage colony-stimulating factor, tumor necrosis factor-α, and IFN-γ by human tumor-infiltrating lymphocytes after autologous tumor stimulation. J Immunol 1991;146:3674–81.
28. Fruh K, Yang Y. Antigen presentation by MHC class I and its regulation by interferon γ. Curr Opin Immunol 1999;11:76–81.
29. Caux C, Dezutter-Dambuyant C, Schmitt D, Banchereau J. GM-CSF and TNF-α cooperate in the generation of dendritic Langerhans cells. Nature 1992;360:258–61.
30. Grabbe S, Bruvers S, Lindgren AM, Hosoi J, Tan KC, Granstein RD. Tumor antigen presentation by epidermal antigen-presenting cells in the mouse: modulation by granulocyte-macrophage colony-stimulating factor, tumor necrosis factor α, and ultraviolet radiation. J Leukoc Biol 1992;52:209–17.
31. Simons JW, Jaffee EM, Weber CE, et al. Bioactivity of autologous irradiated renal cell carcinoma vaccines generated by *ex vivo* granulocyte-macrophage colony-stimulating factor gene transfer. Cancer Res 1997;57:1537–46.
32. Zhou X, Jun do Y, Thomas AM, et al. Diverse CD8$^+$ T-cell responses to renal cell carcinoma antigens in patients treated with an autologous granulocyte-macrophage colony-stimulating factor gene-transduced renal tumor cell vaccine. Cancer Res 2005;65:1079–88.
33. Moran CJ, Arenberg DA, Huang CC, et al. RANTES expression is a predictor of survival in stage I lung adenocarcinoma. Clin Cancer Res 2002;8:3803–12.
34. Dorner BG, Scheffold A, Rolph MS, et al. MIP-1α, MIP-1β, RANTES, and ATAC/lymphotactin function together with IFN-γ as type 1 cytokines. Proc Natl Acad Sci U S A 2002;99:6181–6.
35. Wu JD, Higgins LM, Steinle A, Cosman D, Haugk K, Plymate SR. Prevalent expression of the immunostimulatory MHC class I chain-related molecule is counteracted by shedding in prostate cancer. J Clin Invest 2004;114:560–8.
36. Salih HR, Antropius H, Gieseke F, et al. Functional expression and release of ligands for the activating immunoreceptors NKG2D in leukemia. Blood 2003;102:1389–96.
37. Wu X, Li Y, Crise B, Burgess SM. Transcription start regions in the human genome are favored targets for MLV integration. Science 2003;300:1749–51.
38. Huang X, Wilber AC, Bao L, et al. Stable gene transfer and expression in human primary T-cells by the Sleeping Beauty transposon system. Blood 2006;107:483–91.
39. Straathof KC, Pule MA, Yotnda P, et al. An inducible caspase 9 safety switch for T-cell therapy. Blood 2005;105:4247–54.

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