ABSTRACT

The failure of conventional cancer therapy renders glioblastoma an attractive target for immunotherapy. Tumor cells expressing ligands of the activating immunoreceptor NKG2D stimulate tumor immunity mediated by natural killer (NK), γδ T, and CD8+ T cells. We report that human glioma cells express the NKG2D ligands MICA, MICB, and members of the UL16-binding protein family constitutively. However, glioma cells resist NK cell cytolysis because of high MHC class I antigen expression. Plasmid-mediated or adenovirus-mediated overexpression of MICA in glioma cells enhances their sensitivity to NK and T-cell responses in vitro and markedly delays the growth of s.c. and intracerebral LN-229 human glioma cell xenografts in nude mice and of SMA-560 gliomas in syngeneic VMDK mice. Glioma cells forming progressive tumors after implantation of stably MICA-transfected human LN-229 cells lost MICA expression, indicating a strong selection against MICA expression in vivo. Rejection of MICA-expressing SMA-560 cells in VMDK mice resulted in protection against a subsequent challenge with wild-type tumor cells. Finally, the growth of syngeneic intracerebral SMA-560 tumors is inhibited by peripheral vaccination with adenovirus-mediated, MICA-infected irradiated tumor cells, and vaccination results in immune cell activation in the NK and T-cell compartments in vivo. These data commend MICA immunogene therapy as a novel experimental treatment for human malignant gliomas.

INTRODUCTION

Glioblastoma, the most frequent malignant primary brain tumor, carries a poor prognosis, with a median survival after resection, radiotherapy, and chemotherapy of 12 months (1). The lack of effective immune responses to glial tumors inside the central nervous system has been attributed to the immune-privileged status of the brain conferred by the blood-brain barrier, the lack of conventional lymphatics, and the local release of immunosuppressive factors (2–4). However, both lymphocytes and macrophages infiltrate malignant gliomas, indicating the potential for lymphocyte homing and presentation of processed tumor antigens (2).

NKG2D is a C-type, lectin-like homodimeric receptor expressed by human natural killer (NK), γδ T, and CD8+ αβ T cells (5). In the mouse, NKG2D expression is found on NK cells, activated CD8+ T cells, and lipopolysaccharide-stimulated macrophages (6). Ligation of NKG2D may be a critical activating pathway to provide tumor surveillance (7–9). NKG2D interacts with ligands that are not constitutively expressed but are inducibly expressed, including human MHC class I chain-related A (MICA) and MICB, distant cell stress-inducible but are inducibly expressed, including human MHC class I chain-related A (MICA) and MICB, distant cell stress-inducible expression on neurons (28, 29).

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MATERIALS AND METHODS

Cell Lines and Transfectants. The human Sv-FHAS astrocytic cell line was provided by D. Stanimirovic (Institute of Biological Sciences, National Research Council of Canada, Ontario, Ottawa, Canada). The human malignant glioma cell lines were provided by Dr. N. de Tribolet (Centre Hospitalier Universitaire Vaudois, Lausanne, Switzerland). Primary glioblastoma cells were established from freshly resected tumors, cultured in monolayers, and used between passages 4 and 9 (30). The murine glioma cell lines GL261 and SMA-560 were gifts of X. O. Breakefield (Harvard Medical School, Boston, MA) and D. D. Bigner (Duke University Medical Center, Durham, NC). The cells were maintained in DMEM supplemented with 2 mm l-glutamine (Life Technologies, Inc., Paisley, United Kingdom), 10% FCS (Biochrom KG, Berlin, Germany), and penicillin (100 IU/ml/streptomycin (100 μg/ml) (Gibco). NKL cells, kindly provided by M. J. Robertson (Indiana University School of Medicine, Indianapolis, IN), COS-7, VAC-1, and K562 cells (American Type Culture Collection, Rockville, MD) were grown in RPMI 1640 medium supplemented with 15% FCS, 2 mm l-glutamine, 1 mm sodium pyruvate, and penicillin (100 IU/ml/streptomycin (100 μg/ml). Transfections with cDNA clones, RSV.5neo, RSV.5neo-MICA*01 (10, 11) or RSV.5neo-
PatMIC1 (31) were conducted using FuGENE6 (Roche, Mannheim, Germany) and selection with G418 (1 mg/ml). PatMIC1 is the chimpanzee homolog of MICA, which shows 87% homology and was used as a negative control in some experiments (31). Stable, positive isolates were identified by flow cytometry and, to speed up the selection process, were sorted using a FACS Vantage (Becton Dickinson, Heidelberg, Germany).

Reverse Transcription (RT)-PCR. Total RNA was prepared using RNeasy (Qiagen, Hilden, Germany) and transcribed according to standard protocols. The conditions for all standard PCR were: 5 min at 95°C, 30 cycles of 1 min at 95°C, 1 min at 65°C, 1 min at 72°C, and 10 min at 72°C, using the following PCR primers: H60: sense 5'-GATTGGTACAGACTCTTACCTGTGTT-3' and antisense 5'-CAGAACCTTGTTGCTGAGTTGAT-3' (554 bp); RAE-1: sense 5'-ATGGCAACAGACAGTGGCAAG-3' and antisense 5'-CACATCGGAAAATGGCAATGCAAATATTG-3' (748 bp); MICA: sense 5'-GTTGGGCGGCTGCTCGAAGGTGAGAC-3' and antisense 5'-GGCAGGAGCACTCTGGAGTTCG-3' (635 bp); and antisense 5'-CACAGGACGAGCTCTGGAGTTCG-3' (900 bp); ULBP1: sense 5'-CTGACGAACAGGATCCTTTGTA-3' and antisense 5'-TGAAGGGTGTGTTGGCCATGGCCAT-3' (319 bp); ULBP2: sense 5'-CTGCACGAACAGGATCCTTTGTA-3' and antisense 5'-TGAAGGGTGTGTTGGCCATGGCCAT-3' (327 bp); ULBP3: sense 5'-CTGACGAACAGGATCCTTTGTA-3' and antisense 5'-TGAAGGGTGTGTTGGCCATGGCCAT-3' (323 bp) and antisense 5'-TTATGTTTCTGGCAAGTCTTTGTA-3' (232 bp).

Generation of Monoclonal Antibodies. The mouse P815 mastocytoma cell line was transduced using FuGENE6 with full-length cDNA of NKG2DL MICA*01, MICA*04, MICB*02, ULBP1, ULBP2, and ULBP3 or in RSV.5 neo (15). Transfectants were selected with G418. BALB/c mice were immunized either with a mixture of MICA*01, MICA*04, and MICB*02, or ULBP1, ULBP2, and ULBP3-expressing P815 cells. Splenocytes of immunized mice were fused with P3 Ag8.653 myeloma cells. Hybridoma supernatants were tested by indirect immunofluorescence using a FACS calibur (Becton Dickinson) for selective binding to P815-NKG2DL transfectants and to COS cells transiently transfected with the various NKG2DL cDNAs. Hybridomas producing NKG2DL-specific mAbs were subcloned twice, and immunoglobulins were isolated using an isolating kit (Roche). BAMO-1 (IgG1) and BAMO-2 (IgG2a) recognize MICA and MICB; BAMO-1 (IgG1) is MICA specific; BMO-1 (IgG1) is MICB specific; AUMO-1 (IgG1) is ULBP1 specific; BUMO-2 (IgG1) ULBP2 specific; and CUMO-1 (IgM) is ULBP3 specific (32).

Production of Soluble mNKG2D in Insect Cells. Recombinant soluble mNKG2D lacking the NH2-terminal cytoplasmic region and transmembrane domain was produced in Sf9 insect cells (Invitrogen, Karlsruhe, Germany); anti-H-2Kb (BD PharMingen, Heidelberg, Germany); antibodies (mAbs): W6/32, IgG2a pan anti-HLA-A, -B, -C, -E, -G (Biozol, Echingen, Germany); E4-6 (BD PharMingen, Heidelberg, Germany); anti-CD16 (FcγRIII) was assayed with the mAb 3G8, IgG1 anti-CD16-CyChrome (BD PharMingen). Fluorescence was measured in a Becton Dickinson FAC SCALIB. Specific fluorescence indexes (SFIs) were calculated by dividing mean fluorescence obtained with specific antibody by mean fluorescence obtained with control antibody.

Adenoviral Constructs and Transduction. Replication-deficient recombinant adenoviruses (Ad5dE1,3) that had the E1A region replaced by the full-length MICA*01 cDNA (Ad-MICA) were constructed using the AdEasy System, kindly provided by Bert Vogelstein (The Howard Hughes Medical Institute, Johns Hopkins University School of Medicine, Baltimore, MD). Recombinant adenoviral particles were produced in 293 cells (ATCC CRL-1573), and transgene expression was assessed by RT-PCR. Infection of target cells with recombinant viruses was accomplished by exposing cells to different concentrations of adenovirus in PBS containing 10% glycerol for 15 min, followed by the addition of serum-containing medium for 2 days.

Purification of PBL and Isolation of NK and T Cells. Peripheral blood mononuclear cells were derived from healthy donors by density gradient centrifugation (Biocoll, Biochrom KG) and depletion of plastic-adherent cells. Peripheral blood lymphocytes (PBLs) were cultured on irradiated RPMI 8866 feeder cells to obtain polyclonal NK cell populations. To further enrich NK cells, PBLs were sorted by immunomagnetic depletion using Dynabeads (NK Cell Negative Isolation Kit; Dynal, Oslo, Norway). CD3+ CD56+ cells were used for cytotoxicity assays. To obtain purified T cells, fresh PBLs were depleted of B cells and monocytes using the LympHere Kit (One Lambda, Inc., Canoga Park, CA).

Mouse Lymphocyte Isolation. Murine NK cells were prepared from splenocytes from CD1-deficient BALB/c nude mice or MVD mice. NK cells were positively selected using DX5 mAb-coupled magnetic beads with the corresponding column system (Miltenyi Biotech, Bergisch Gladbach, Germany). Polyclonal mouse NK cells were cultured with mouse IL-2 (5000 units/ml; Preprotech, London, United Kingdom) for at least 10 days before use in cytotoxicity assays.

Cytotoxicity Assay. Cytotoxicity was assayed in 4-h 51Cr release assays in the absence or presence of various mAbs or soluble mNKG2D. The concentration for the masking experiments was 10 µg of mAb/ml and 20 µg/ml for soluble mNKG2D. The 51Cr release assay was performed using 2000 51Cr-labeled targets/well. Effector and target cells were incubated at various E/T ratios for 4 h. Spontaneous release of 51Cr was determined by incubating the target cells with medium alone. Maximum release was determined by adding NP40 to a final concentration of 2%. The percentage of 51Cr release was calculated as follows: 100 × (experimental release – spontaneous release)/ [maximum release – spontaneous release].

T-Cell Reaction against Allogeneic Glialoma Cells. To suppress LN-229 glioma cell proliferation, the cells were irradiated at 100 Gy. HLA-A2-positive T cells or peripheral blood mononuclear cells (106/well) were cocultured with 105 irradiated LN-229 HLA-A2-negative glioma cells in 96-well plates in triplicates. After 4 days the cells were pulsed for 24 h with [3H]-thymidine (Amersham Pharma Biotech, Arlington Heights, IL) and harvested with a cell harvester (Tomtec, Hamden, CT). Incorporated radioactivity was determined in a Wallac 1450 Microbeta Plus Liquid Scintillation Counter. For the priming assays, nonirradiated glioma cells were incubated with peripheral blood mononuclear cells or T cells for specific priming and clonal expansion. Lymphocytes were harvested on day 5 and used as effectors in a 4-h 51Cr release cytotoxicity assay. Mock-transfected LN-229.neo cells or LN-229 cells transfected with MICA*01 or PatMIC1 were used as targets.

Interleukin (IL)-2 Release Assay. T cells (1 × 106/well) isolated at day 10 after tumor inoculation from spleens of vaccinated or control animals were stimulated ex vivo with irradiated SMA-560 cells (1 × 106). T-cell supernatants from triplicate wells were collected and pooled 48 h later. IL-2 release was determined by ELISA (R&D Systems, Minneapolis, MN).

Mice and Animal Experiments. Athymic CD1-deficient BALB/c nude nude mice were purchased from Charles River Laboratories (Sulzfeld, Germany), and MVD mice were purchased from the TSE Research Center (Berkshire, United Kingdom). Mice of 6–12 weeks of age were used in all experiments. The experiments were performed according to NIH guidelines, Guide for the Care and Use of Laboratory Animals. Groups of 4–6 mice were injected s.c. in the right flank with transfected LN-229 tumor cells or uninfected or infected SMA-560 glioma cells (1 × 106 cells) in 0.1 ml of PBS, as indicated. Mice were examined regularly for tumor growth using a metric caliper and killed when tumors reached >12-mm diameter. Mice were anesthetized by i.p. injection of 7% chloral hydrate before all intracranial procedures. For intracranial implantation, the mice were placed in a stereotactic fixation device.
or 5

MICB, ULBP1, ULBP2, and ULBP3 by glioma cells. 12 glioma cell

Germany) was introduced to a depth of 3 mm. Five x 10⁴ LN-229 glioma cells

expressed MICA, ULBP2, and ULBP3. MICB was expressed by 9 of

NKG2DL and MHC class I antigens using mAbs. All glioma cell lines

in Sv-FHAS cells. We next monitored the cell surface expression of

glioma cell cultures. In contrast, none of the NKG2DLs were detected

not detectable in U251MG or U373MG cells and several primary

all cell lines and most of the primary glioma cell cultures. MICB was

and ULBP1, ULBP2, and ULBP3 mRNA were expressed by almost

vivo

cultured glioma cells were assessed by RT-PCR (Fig. 1). MICA

survival patterns in mice bearing intracerebral gliomas was performed by the

two-tailed Student

t

P

P

0.05 considered significant and

0.01

considered highly significant (Excel; Microsoft, Seattle, WA). Evaluation of

survival patterns in mice bearing intracerebral gliomas was performed by the

Kaplan-Meier method (33), and Ps were evaluated by the Mantel log-rank test

(34).

RESULTS

Expression of NKG2DL and MHC Class I Antigens by Human
Gloma Cells. To investigate the expression of the NKG2DL MICA, MICB, ULBP1, ULBP2, and ULBP3 by glioma cells, 12 glioma cell lines, a nonneoplastic astrocyte line, Sv-FHAS, and five primary ex
vivo cultured glioma cells were assessed by RT-PCR (Fig. 1). MICA and ULBP1, ULBP2, and ULBP3 mRNA were expressed by almost all cell lines and most of the primary glioma cell cultures. MICB was not detectable in U251MG or U373MG cells and several primary glioma cell cultures. In contrast, none of the NKG2DLs were detected in Sv-FHAS cells. We next monitored the cell surface expression of NKG2DL and MHC class I antigens using mAbs. All glioma cell lines expressed MICA, ULBP2, and ULBP3. MICB was expressed by 9 of 12 cell lines, whereas ULBP1 was only detectable for 5 of 12 cell lines. All of the primary gliomas expressed MICA and ULBP2, and the other NKG2DL at various levels (Table 1). MICB was expressed by TU132, TU140, and TU203, whereas TU113 and TU207 did not (Table 1). All cell lines and primary cultures showed high levels of
MHC class I antigen expression. The nonneoplastic Sv-FHAS cell line did not express any NKG2DL, as determined by RT-PCR and flow cytometry. Sv-FHAS cells showed low MHC class I antigen expression (Table 1).

Stable Transfection and Adenoviral Gene Transfer of MICA
into Glioma Cells Stimulate NK Cytolytic Activity. To determine whether increased NKG2DL expression confers additional stimulatory signals for NK cells, we generated stable MICA*01 and PatrMIC1 [chimpanzee MIC homolog (31)] transfectants of the LN-
229 and Sv-FHAS cell lines (Fig. 2, A and B). PatrMIC1 was included in this study to serve as a negative control for the nude mouse model in vivo (see below). Enhanced MICA or PatrMIC1 expression rendered cells susceptible to lysis by human NKL effector cells, suggesting that ectopic MIC expression overcomes the inhibitory effect of MHC class I antigens (Fig. 2, A and B, lower panels). Similar results were obtained with freshly isolated polyclonal NK cells (data not shown). For a gene therapeutic approach, we generated an adenoviral vector encoding MICA*01 (Ad-MICA). Infection of LN-229, primary glioma cells (Fig. 2C), or other glioma cell lines (data not shown) with Ad-MICA resulted in a marked increase in MICA expression. Adenoviral MICA gene transfer also promoted NK-mediated cytolysis (Fig. 2, lower panel).

For blocking experiments with mAbs, we first ruled out that a potential lysis seen in the presence of mAbs is attributable to antibody-dependent cellular cytotoxicity (ADCC). Unlike fresh polyclonal NK cells isolated from PBLS, the NKL cell line used here does not express FcγRIII (CD16), which mediates ADCC (Fig. 3A; Ref. 35). LN-229.neo transfectants were sensitized to NK cytolysis by treatment with the anti-MHC class I mAb W6/32 (Fig. 3B), confirming that classical MHC class I antigens protect LN-229.neo cells from NK cytolysis. Anti-MICA/B (BAMO-1) or anti-NKG2D (M585) mAb had no effect on cytolysis in LN-229.neo cells. However, these mAbs either attenuated (anti-MICA) or blocked (anti-NKG2D) the sensitization mediated by anti-MHC class I mAb. The superior effect of anti-NKG2D compared with anti-MICA reflects the composition of the system of a single receptor (NKG2D) in NK cells but multiple ligands (MIC and ULBP molecules) expressed by LN-229 cells (Fig. 1; Table 1). Soluble mouse MICA (mMICA) attenuated the anti-MHC class I mAb-mediated sensitization as effectively as anti-MICA, suggesting that murine NKG2D interacts with some human NKG2DL. NK cytolysis of LN-229.MICA targets pretreated with anti-MICA or

anti-NKG2D mAb was reduced (Fig. 3C), demonstrating that enhanced cytolysis of LN-229.MICA cells is attributable to MICA overexpression, overruling MHC class I antigen inhibitory signals. Similar results were obtained with LN-229.PatrMIC1 transfectants (Fig. 3D).

Costimulatory Functions of MICA-transfected Glioma Cells.
Allostimulation assays were performed to analyze whether MICA/ NKG2D interactions promote the induction of T-cell responses. LN-229.MICA or LN-229.PatrMIC1 cells were substantially more potent stimulators (3-fold) of T-cell proliferation than LN-229.neo cells (Fig. 4A). mAb masking of MICA abrogated the augmentation of T-cell proliferation mediated by MICA but had no effect when T cells were coincubated with LN-229.neo cells (Fig. 4B). Additional evidence for the costimulatory potential of NKG2D for T cells was obtained by rechallenging primed T cells. T cells were primed for 4 days with LN-229.neo, LN-229.MICA, or LN-229.PatrMIC1 cells and then used as effectors against fresh LN-229.neo, LN-229.MICA, or LN-229.PatrMIC1 cells. No target cell lysis was seen when LN-229.neo...
cells, LN-229.MICA, or LN-229.PatrMIC1 cells were used as targets for LN-229.neo-primed T cells (Fig. 4, C–E). In contrast, LN-229.MICA-primed or LN-229.PatrMIC1-primed T cells efficiently killed LN-229.neo, LN-229.MICA, or LN-229.PatrMIC1 cells with comparable efficacy, indicating that NKG2D ligation by MICA or PatrMIC1 acts as a costimulatory signal in the priming phase of T cells (Fig. 4, C–E).

**MICA Expression Delays Growth of Glioma Xenografts in Nude Mice.** We next sought to examine the immune-stimulatory capacity of MICA-overexpressing glioma cells in *in vivo* models. We first tested the physical and functional engagement of MICA*01 and PatrMIC1 by mouse NKG2D (mNKG2D). Interaction of MICA*01 and PatrMIC1, respectively, with mNKG2D, was assessed by using soluble mNKG2D. COS-7 cells transiently transfected with either MICA*01 or PatrMIC1 cDNA stained with the mAb AMO-1, but only MICA*01-transfected COS cells bound soluble mNKG2D (data not shown). We concluded that mNKG2D interacts with human MICA*01 but not with PatrMIC1. PatrMIC1 differs from MICA and MICB in several positions directly involved in NKG2D interaction, which may selectively interfere with binding of mNKG2D (10–12). Accordingly, NK cell cytotoxicity assays using polyclonal mouse NK cells as effector cells revealed marked lysis of human MICA glioma cell transfectants but not of mock or PatrMIC1 transfectants (Fig. 5A), indicating an activation by MICA*01 of the mouse NKG2D receptor. PatrMIC1 transfectants served hereafter as negative controls in the ensuing nude mice studies.

The immune-stimulatory potential of MICA-transfected LN-229 cells for the NK cell-mediated, anti-glioma response in *in vivo* was examined in s.c. and intracerebral xenograft glioma models in nude mice that have NK cells but lack T cells. LN-229 glioma cells were injected s.c., and the tumor sizes were measured every 2 days. LN-229.PatrMIC1 cells grew rapidly to form compact tumors, whereas LN-229.MICA tumor growth was significantly delayed (Fig. 5B). Tumor volumes differed significantly (t test, *P < 0.01*) from day 17 to the end of the experiment. When LN-229 cells were implanted stereotactically into the brains of nude mice, animals carrying LN-229.PatrMIC1 tumors developed neurological symptoms and had to be sacrificed at days 20–21. In contrast, animals carrying LN-229.MICA tumors showed significantly prolonged survival (Fig. 5C; log-rank test, *P < 0.01*). Appropriate control experiments disclosed no difference between the proliferation of LN-229.MICA and LN-229.PatrMIC1 cell lines in *vitro* (Table 2). Because MICA gene transfer delayed glioma growth but did not lead to tumor rejection, we characterized the glioma cells forming progressive tumors in nude mice. To this end, we isolated the tumor cells, which had grown s.c., and analyzed the expression of MICA and PatrMIC1 by flow cytometry. These experiments yielded two findings that support a proficient role for MICA as a potent molecule inhibiting the growth of gliomas: (a) glioma cells recovered from mice that had received LN-229.PatrMIC1 cells showed unaltered levels of high PatrMIC1 expression before and after tumor inoculation, consistent with the failure of PatrMIC1 to interact with mNKG2D (Fig. 5D, left); (b) all tumor cells harvested from mice that had received LN-229.MICA*01 cells were essentially devoid of MICA. This is probably because of tumor formation by a small non-MICA-expressing population among the LN-229.MICA cells (Fig. 5D, right). Endogenous MICA expression in LN-229.PatrMIC1 cells was also reduced after glioma cell passaging *in vivo*: the SFI decreased from 2.6 before inoculation to 1.4 after inoculation. MHC class I antigen expression measured by flow cytometry did not change after MICA transfection or *in vivo* passaging (data not shown).

**NKG2DL Expression by Mouse Glioma Cell Lines.** We evaluated two syngeneic mouse glioma cell models, SMA-560 cells in VM/Dk mice (spontaneous tumor) and GL261 cells in C57BL/6 mice (3-methylcholanthrene induced), for a therapeutic trial of MICA gene transfer *in vivo*. The mNKG2DL RAE-1β was prominently expressed by SMA-560 cells, whereas GL261 showed only a weak expression. H60 was only expressed by SMA-560 and not by GL261 cells, consistent with the report that C57BL/6 mice do not express H60 (Ref. 8; Fig. 6A). Soluble mNKG2D was more prominently bound by SMA-560 than GL261 cells (Fig. 6B), indicating higher expression levels of mNKG2DL in SMA-560 cells. Whereas SMA-560 cells expressed both MHC class I antigens H-2k and H-2d, which are potential ligands for inhibitory Ly49 receptors, GL261 cells showed only low level H-2d expression (Fig. 6B). SMA-560 cells thus resemble the phenotype of human glioma cell lines and were used in further animal experiments. When SMA-560 cells were infected with Ad-MICA, MICA expression increased in a multiplicity of infection (MOI)-dependent manner: the SFI values were 6.6 at 100 MOI, 12.2 at 300 MOI, and 22.8 at 1000 MOI (Fig. 7B). Similar to LN-229 cells, the growth of SMA-560 cells was unaffected by forced MICA expression (Table 2). The functional significance of increased MICA expression on mouse glioma cells after Ad-MICA infection was confirmed in cytotoxicity assays using syngeneic VM/Dk NK cells as effectors (Fig. 6C). Blocking experiments with anti-MICA mAb and soluble mNKG2D demonstrated that the increased killing of Ad-

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**Table 1** Flow cytometric analysis of MHC class I antigens and NKG2DL expression by human malignant glioma cell lines, non-neoplastic Sv-FHAS astrocytes, and primary glioma cell cultures. The cells were stained with the indicated mAb or isotype-matched Ig. Expression was quantified as SFI values (mean fluorescence specific mAb /mean fluorescence isotyp control).

<table>
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<tr>
<th>Cell line</th>
<th>MHC class I (W6/32)</th>
<th>MICA (AMO-1)</th>
<th>MICB (BMO-1)</th>
<th>MICA/B (AMO-1)</th>
<th>ULBP1 (AUO-1)</th>
<th>ULBP2 (BMO-2)</th>
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<td>7.4</td>
<td>1.3</td>
<td>1.8</td>
<td>1.0</td>
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Fig. 2. Ectopic MICA*01 and PatrMIC1 expression by glioma cells promote NK cell-mediated cytolysis. LN-229 (A) or Sv-FHAS (B) cells transfected with MICA*01 or PatrMIC1 were assessed for MICA (mAb BAMO-1) or PatrMIC1 (mAb BAMO-2) expression (filled profiles) as compared with mock transfectants (neo-control) by flow cytometry. Open profiles show the labeling with isotype-matched immunoglobulin. The SFI values are indicated in the upper right of each panel. C, LN-229 or TU113 primary glioma cells were infected with Ad-MICA or Ad-dE1 (300 MOI) and assessed for MICA expression accordingly. In the lower panels of A–C, the cell lines were tested as targets for human NKL cells in 51Cr-release assays. Data are expressed as specific lysis at different E:T ratios (means; bars, SD). t test: *, P < 0.05; **, P < 0.01.
MICA-infected SMA-560 cells was attributable to MICA expression. Attenuation of the constitutive lysis of control-infected SMA-560 cells by mNKG2D probably reflects the activation by endogenous NKG2DLs such as RAE-1 and H60 (Fig. 6D). Accordingly, neutralization of MICA was less effective in inhibiting lysis of Ad-MICA-infected cells than mNKG2D.

MICA Expression in SMA-560 Glioma Cells Delays Tumor Growth. To analyze the immune-stimulatory capacity of MICA-transduced SMA-560 tumor cells in vivo, VMDk mice were inoculated s.c. with syngeneic tumor cell transductants. There was no difference between the in vivo growth of Ad-dE1-infected and uninfected SMA-560 cells. To analyze the immune-stimulatory capacity of MICA-transduced SMA-560 tumor cells in vivo, VMDk mice were inoculated s.c. with syngeneic tumor cell transductants. There was no difference between the in vivo growth of Ad-dE1-infected and uninfectected SMA-560 cells.

Fig. 3. NKG2DL and MHC class I antigen expression determines NK cytolysis. A, the NKL cell line and fresh polyclonal NK cells were assessed for CD16 (FcγRIII) expression by flow cytometry, which might mediate ADCC in mAb blocking experiments. Open profiles show the labeling with isotype-matched immunoglobulin. The SFI values are indicated in the upper right of each panel. B-D, different target cell lines were pretreated with control immunoglobulin or anti-MICA BAMO-1 mAb, anti-PatrMIC1 BAMO-2 mAb, anti-MHC class I W6/32 mAb, or soluble mNKG2D. NKL effector cells were pretreated with control immunoglobulin or anti-NKG2D M585 mAb for 30 min before use in 51Cr-release cytotoxicity assays. LN-229.neo (B), LN-229.MICA (C), or LN-229.PatrMIC1 (D) were used as targets. The specific lytic activities are given for an E:T ratio of 40:1 (means; bars, SD). t test: *, P < 0.05; **, P < 0.01, compared with isotype control immunoglobulin.

Fig. 4. Modulation of alloreactivity to glioma cells by MICA and PatrMIC1. A, irradiated LN-229.neo, LN-229.MICA, or LN-229.PatrMIC1 cells were coincubated with HLA-A2-mismatched T cells. [3H]Thymidine was added on day 4 for 16 h, and incorporation was measured by liquid scintillation counting. B, irradiated glioma cells were preincubated with isotype control antibody or anti-MICA (BAMO-1) and processed as in A. Data are expressed as cpm (bars, SD). t test: **, P < 0.01, compared with LN-229.neo in A or isotype control antibodies in B. C-E, T cells were incubated with glioma cells, harvested on day 4, and used as effectors in a 4-h 51Cr-release assay at the indicated E:T ratios (means; bars, SD). t test: *, P < 0.05; **, P < 0.01.
MICA/NKG2D-MEDIATED THERAPY OF GLIOMAS

DISCUSSION

This study investigates the potential of NKG2DL to promote an immune response to glioblastoma. We show that NKG2D-mediated

Table 2 MICA gene transfer does not modulate glioma cell growth as assessed by [3H]thymidine incorporation in vitro

<table>
<thead>
<tr>
<th>Proliferation (cpm)</th>
<th>LN-229</th>
<th>SMA-560</th>
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<tbody>
<tr>
<td>Parental</td>
<td>1802 ± 401</td>
<td>3078 ± 523</td>
</tr>
<tr>
<td>PatrMIC1 (stable)</td>
<td>1766 ± 431</td>
<td>1722 ± 386</td>
</tr>
<tr>
<td>MICA (stable)</td>
<td>1766 ± 431</td>
<td>1722 ± 386</td>
</tr>
<tr>
<td>Ad-dE1 (300 MOI)</td>
<td>3122 ± 489</td>
<td>3122 ± 489</td>
</tr>
<tr>
<td>Ad-MICA (300 MOI)</td>
<td>3122 ± 489</td>
<td>3122 ± 489</td>
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</tbody>
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Fig. 5. Ectopic MICA expression delays the growth of s.c. and intracerebral human glioma xenografts in nude mice. A, LN-229 transfectants were tested as targets for BALB/c nude mice NK cells in a 51Cr release assay (means; bars, SD). t test: **, P < 0.01. B, the growth of s.c. LN-229.PatrMIC1 or LN-229.MICA tumors was monitored every 2 days (mean; bars, SD); t test: **, P < 0.01. C, LN-229.PatrMIC1 (solid line) or LN-229.MICA*01 (broken line) cells (5 × 10⁴) were inoculated intracerebrally in BALB/c nude mice. Survival data for five animals per group are shown, evaluated by the Kaplan-Meier method (log-rank test, P < 0.01). D, at 25 days after injection of tumor cells, the mice were killed, and freshly isolated tumor cells from s.c. tumors were analyzed for PatrMIC1 in PatrMIC1 tumors (left) or MICA in MICA tumors (right) by flow cytometry. Representative tumor isolates are shown as gray profiles, the preinoculation levels as black profiles, and an isotype control as open profiles.
MICA/NKG2D-MEDIATED THERAPY OF GLIOMAS

NK cell triggering induced by NKG2DLs expressed on glioma cells is overruled by MHC class I antigens that engage inhibitory receptors on NK cells. Gene transfer-mediated ectopic expression of MICA on human and murine glioma cells permits their in vitro immune recognition and lysis and in vivo growth inhibition, despite a high level MHC class I antigen expression. These observations provide a rationale for MICA-based immunogene therapy of gliomas.

Malignant transformation of glial cells is associated with an NKG2DL expression, i.e., of MICA/B and ULBP molecules, at the cell surface (Fig. 1; Table 1). However, the NK cell inhibitory potential of MHC class I antigens expressed on human glioma cells masks the activating signals of NKG2DL. The interaction of effector NKG2D with endogenous tumor MIC and ULBP molecules promotes NK cell-mediated tumor cell lysis in vitro when MHC class I antigens are masked (Fig. 3B). NK cell-mediated tumor cell lysis was also achieved when MICA expression was enhanced via plasmid transfection or adeno viral gene transfer (Figs. 2 and 3C; Ref. 27). Thus, glioma cells outbalance activating ligand expression by inhibitory MHC class I antigens and prevent NK cell lysis. This concept is supported by a highly significant correlation between MHC class I antigen expression and NKG2DL expression on human glioma cells when the SFI values for the NK cell inhibitory MHC class I antigens were correlated with a sum score of the SFI values for MICA, MICB, ULBP1, ULBP2, and ULBP3 (t test, r² = 0.6523, P = 0.0015).

One way of immune escape for glioma cells may be the expression of MHC class I antigens at a level that allows sufficient binding of NK inhibitory receptors and escape from NK-mediated killing. High expression of MHC class I antigens in human glioblastomas in vivo has been confirmed by several studies (37, 38). MHC class I down-regulation, conversely, is a hallmark of many peripheral cancers and decreases the susceptibility of tumor cells to lysis by cytotoxic T cells (26, 39). Possibly only those glioma cells that show an up-regulation of MHC class I antigens expression to compensate for the acquisition of activating NKG2DL expression during malignant transformation will survive. This selection may render tumor cells more vulnerable toward T cell-dependent elimination through tumor-associated antigen detection (26, 39). Because the brain lacks specific brain-associated lymphoid tissue, antigens that are introduced directly into the brain parenchyma exclusively elicit transient innate inflammatory immune responses but no adaptive immunity (40–44). However, if immunization is elicited by injecting the identical antigen outside the brain, the adaptive immune system becomes primed, and antigenic epitopes anywhere within the brain will be recognized as targets of either activated effector T cells, B cells, or antibodies (40, 42, 44). This immunological peculiarity may explain high expression levels of MHC class I antigens on glioma cells that can effectively present tumor-associated antigens to primed T cells. Because intracerebral T-cell priming is not found, the MHC class I antigens may only serve to suppress NK cell activation by NKG2DL.

The highly lethal nature of glioblastoma suggests that the levels of activating NKG2DLs expressed by glioma cells are too low to induce antitumor immunity. Thus, we concluded that immunity to gliomas may be boosted by engineering cells expressing high levels of activating NKG2DL. We noted that MICA gene transfer induces NK and T-cell responses to glioma cells in vitro (Figs. 2–4) and delays growth in a human xenograft and syngeneic mouse glioma model in vivo (Figs. 5–7). In this model, the in vivo selection for low MICA-expressing glioma cells strikingly demonstrated the NK cell activating potential of the NKG2D/NKG2DL system (Figs. 5D and 7B). Previously, it was shown that ligation of NKG2D on NK and T cells can promote subsequent T-cell immunity to parental tumors that lack NKG2DL, although T-cell priming was NK cell independent (8). However, a similar study did not detect such T cell-mediated memory

Fig. 6. SMA-560 glioma cells in VMDk mice as syngeneic model for MICA gene transfer. A, expression of the murine NKG2DL RAE-1β and H60 mRNA was assessed by RT-PCR. YAC-1 cells were used as a positive control. β-actin was used as a standard. B, NK cell-mediated tumor cell lysis was assessed by a 51Cr-release assay at E:T ratios of 40:1 (means; bars, SD). **, P < 0.01; t test:** *, P < 0.05; ***, P < 0.001. C, uninfected, Ad-dE1 (300 MOI)-infected, and H60 mRNA was assessed by -actin RT-PCR. YAC-1 cells were used as a positive control. B, NK cell-mediated tumor cell lysis was assessed by a 51Cr-release assay at E:T ratios of 40:1 (means; bars, SD). **, P < 0.01; t test:** *, P < 0.05; ***, P < 0.001.
Fig. 7. MICA delays SMA-560 glioma growth in syngeneic mice. A, the growth of s.c. tumors formed by SMA-560 cells infected in vitro with Ad-dE1 (1000 MOI) or Ad-MICA (100, 300, or 1000 MOI) was monitored every 2 days. Two of 6 animals challenged with Ad-MICA-infected cells at 300 and 1000 MOI did not develop a measurable tumor. B, SMA-560 cells infected with Ad-MICA at increasing MOI were assessed for MICA expression before subcutaneous inoculation (black profiles). Open profiles show the labeling with isotype-matched immunoglobulin. Furthermore, glioma cells were freshly isolated from s.c. tumors at 10 days after inoculation and reanalyzed for MICA expression (gray profiles). C, VMDk mice that had previously rejected MICA-transduced SMA-560 cells were inoculated s.c. with wild-type SMA-560 glioma cells in the opposite flank. Primary exposure preceded the challenge by 10 weeks (means; bars, SD). t test: **, P < 0.01. D, 5 x 10^3 SMA-560 cells were inoculated intracerebrally in syngeneic VMDk mice (day 1). At days 3 and 8, the animals were vaccinated s.c. with 1 x 10^6 irradiated Ad-dE1- or Ad-MICA-infected (1000 MOI) or uninfected SMA-560 cells. The graph shows survival data for 6 animals/group, evaluated by the Kaplan-Meier method (log-rank test, P < 0.01). E and F, at day 10, splenocytes were recovered from the differently vaccinated animals, and T and NK cells were isolated. E, NK cells were used as effector cells in a 51 Cr release assay using YAC-1 cells as targets (means; bars, SD). t test: *, P < 0.05; **, P < 0.01. F, isolated T cells were restimulated with irradiated wild-type SMA-560 cells, and IL-2 release was measured by ELISA 48 h later.
against the parental tumor in mice (7). Our data support that NKG2D engagement costimulates human CTL responses because prior immunization with tumor cells expressing the NKG2DL MICA induces protective immunity against a challenge with wild-type glioma cells in mice that had previously rejected MICA-transduced glioma cells (Fig. 7C; Ref. 8, 45). Finally, the peripheral vaccination with irradiated Ad-MICA-infected cells after the intracerebral implantation of wild-type tumor cells delayed the growth of syngeneic gliomas and promoted immune activation in the NK and T-cell compartments (Fig. 7, E and F, and Fig. 8). NKG2DL expression and consequent activation of NK cells and T cells may thus provide a novel therapeutic approach for the treatment of human gliomas. If the magnitude of the effect achieved with peripheral vaccination, i.e., a median prolongation of survival from 27.5 in control animals to 46 days in SMA-560.Ad-MICA-vaccinated animals, with 2 of 6 animals (33%) alive and asymptomatic after 90 days (Fig. 7D), could be transferred into the human situation, this would be a major advance, compared with all chemotherapy trials performed in the recent 20 years (46).

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