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HER-2, gp100, and MAGE-1 Are Expressed in Human Glioblastoma and Recognized by Cytotoxic T Cells

Gentao Liu,[1] Han Ying,[1] Gang Zeng,[2] Christopher J. Wheeler,[1] Keith L. Black,[1] and John S. Yu[1]

[1]Maxine Dunitz Neurosurgical Institute, Cedars-Sinai Medical Center, Los Angeles, California, and [2]Department of Urology, David Geffen School of Medicine, University of California, Los Angeles, California

ABSTRACT

It has recently been demonstrated that malignant glioma cells express certain known tumor-associated antigens, such as HER-2, gp100, and MAGE-1. To further determine the possible utilization of these antigens for glioma immunotherapy and as surrogate markers for specific tumor antigen cytotoxicity, we characterized the presence of mRNA and protein expression in 43 primary glioblastoma multiforme (GBM) cell lines and 7 established human GBM cell lines. HER-2, gp100, and MAGE-1 mRNA expression was detected in 81.4%, 46.5%, and 39.5% of the GBM primary cell lines, respectively. Using immunoreactive staining analysis by flow cytometry, HER-2, gp100, and MAGE-1 protein expression was detected in 76%, 45%, and 38% of the GBM primary cell lines, respectively. HLA-A1-restricted epitope specific for MAGE-1 peptide (EADPTGHSY) CTL clone B07 and HLA-A2-restricted epitope specific for HER-2 peptide (KIFGSLAFL) CTL clone A05 and gp100 peptide (ITDQVPFSV) CTL clone CK3H6 were used in this study. The specificity of CTL clone was verified by HLA/peptide tetramer staining. Three CTL clones could efficiently recognize GBM tumor cells in an antigen-specific and MHC class I-restricted manner. IFN-γ treatment can dramatically increase MHC class I expression of GBM tumor cells and significantly increase CTL recognition of tumor cells. Treatment with the DNA hypomethylating agent 5-aza-2′-deoxycytidine induced and up-regulated the mRNA expression of MAGE-1 and epitope presentation by autologous MHC. These data indicate that HER-2, gp100, and MAGE-1 could be used as tumor antigen targets for surrogate assays for antigen-specific CTLs or to develop antigen-specific active immunotherapy strategies for glioma patients.

INTRODUCTION

The incidence of primary brain tumors in the United States is approximately 17,400 per year. The most common and deadly brain tumor type is glioblastoma multiforme (GBM). The survival rate for this disease per year has not changed substantially over the past 50 years, despite advances in surgery, radiotherapy, and chemotherapy (1, 2). Clearly, novel strategies are needed to significantly affect the outcome for these patients. In melanoma patients, adoptive transfer of autologous tumor-infiltrating lymphocytes with interleukin (IL)-2 resulted in tumor regression, suggesting that the immune system plays a critical role in fighting tumors (3). To further understand the molecular nature of tumor antigens recognized by T cells *in vivo*, much effort has been devoted to the identification of tumor antigens that may serve as important immune targets.

MAGE-1 was initially analyzed from melanomas and became the first identified tumor antigen recognized by T cells (4). The dominant epitope, EADPTGHSY, was subsequently identified and recognized by CTLs in the context of HLA-A1 (5). MAGE-1 is expressed in 48% of metastatic melanomas and in many different histological tumor types (6–9). MAGE-1, however, is silent in normal cells except for testis and placenta (4). Thus far, at least 11 MHC class I epitopes and two MHC class II epitopes have been identified (10). The vaccination of MAGE-1 peptide-pulsed dendritic cells (DCs) is capable of inducing clinical and systemic tumor-specific immune responses without provoking major side effects in melanoma patients (11). Melanoma patients immunized with melanoma cell vaccine induce antibody responses to recombinant MAGE-1 antigen (12).

Human melanoma-associated antigen, gp100, is a melanocyte differentiation antigen recognized in patients with melanoma by HLA-restricted CTLs and antibody (13, 14). It appears to be a promising target antigen. Several clinical trials indicated that gp100 was a highly immunogenic antigen in melanoma patients and also found a strong correlation between T-cell recognition of the gp100 antigen and clinical responses (15, 16). Thus far, at least 16 MHC class I-restricted gp100 epitopes and several MHC class II epitopes have been identified by CTLs derived from different patients (10). gp100 ($9_{209-217}$) ITDQVPFSV is the most well characterized and most commonly used epitope in preclinical and clinical melanoma studies.

HER-2, also called HER-2/neu or c-erbB2, encodes a 185-kDa transmembrane glycoprotein with tyrosine-specific kinase activity and extended homology in structure and sequence to epidermal growth factor receptor (17). The HER-2 oncogenic protein is a well-defined tumor antigen. Gene amplification and overexpression of HER-2 have been demonstrated with high frequency in a number of human malignancies including breast and ovarian tumors (18), as well as in colon carcinoma (19) and renal cell carcinoma (20). Patients with HER-2 protein-overexpressing breast, ovarian, non-small cell lung, colon, and prostate cancers have been shown to have a pre-existent immune response to HER-2. HER-2-specific vaccine has been tested in human clinical trials. Early results demonstrate that the immunity elicited is durable even after vaccinations have ended (21). A number of HER-2-specific CTLs have been isolated that could recognize not only HER-2-overexpressing ovarian and breast carcinoma but also other epithelial tumors. Due to the overexpression of HER-2 in tumor cells and the immunogenicity of HER-2, it represents an excellent target for T-cell-mediated immunotherapy (22). Thus far, at least 13 HLA class I epitopes and several HLA class II epitopes have been identified (10). The dominant HLA-A2-restricted epitope derived from HER-2 extracellular domain ($9_{369-377}$) KIFGSLAFL (23) will be investigated in this study.

Although there were reports of HER-2 protein expression (24–26) and gp100 and MAGE-1 mRNA expression (27–30) in brain tumor, the immunogenicity of HER-2, gp100, and MAGE-1 and regulation in GBM are still unknown. In this study, we analyzed HER-2, gp100, and MAGE-1 mRNA expression by reverse transcription-PCR (RT-PCR) and protein level by flow cytometry analysis. We also investigated whether HER-2, gp100, and MAGE-1 could be naturally processed and could then present their dominant epitopes to CTLs *in vitro*. We demonstrate that the majority of GBMs express these antigens and process the dominant epitope, thus allowing CTL recognition of these peptides. Finally, we investigated whether IFN-γ or DNA hypomethylating agent treatment could affect CTL recognition of tumor cells. These findings may allow surrogate assay determination of specific tumor antigen cytotoxicity for glioma immunotherapy. The characterization of these tumor-associated antigens will also

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Requests for reprints: John S. Yu, 8631 West Third Street, Suite 800E, Los Angeles, CA 90048. Phone: (310) 423-0845; Fax: (310) 423-0810; E-mail: Yuj@cshs.org.

allow development of antigen-specific active and adoptive immunotherapy strategies.

MATERIALS AND METHODS

Tumor Specimens and Primary Cell Culture. Brain tumor specimens from patients were obtained from the Cedars-Sinai Institutional Review Board-approved Brain Tumor Registry after being reviewed and released by a pathologist in the operating room. Classification of tumor type and grade was made by independent pathologists in accordance with the WHO histological typing of central nervous system tumors. All tumor specimens were obtained from patients who had signed institutional review board-approved informed consent forms. The glioma tissues were processed under sterile conditions in a laminar flow hood by certified technicians. Tumor cells were recovered from Ficoll-Hypaque gradients (Invitrogen, Carlsbad, CA), washed in tissue culture medium, and aliquoted for tissue culture. For the establishment of primary tumor cell line culture, tumor cells were seeded in a flask in the following culture medium: Ham's F-12/DMEM with high glucose (Irvine Scientific, Santa Ana, CA), 10 mM HEPES (Invitrogen), 0.1 mg/ml gentamicin (Invitrogen), and 10% heat-inactivated fetal bovine serum (Irvine Scientific). Primary tumor cells were subcultured for three to four passages. Each passage yielded a 2–4-fold increase in cell number. Tumor cell culture followed the Standard Operating Procedure approved by Cedars-Sinai Biosafety Committee. Four patients' cells were randomly selected for chromosomal karyotype analysis at the Cytogenetics Laboratory Facility, Cedars-Sinai Medical Center. At least 20 cells were analyzed from the cultures of each of these four patients. Four of these karyotypes displayed gain of chromosome 7. The criteria of karyotypic analysis was based on the International System of Cytogenetic Nomenclature in 1991 (31).

Cell Lines. GBM cell lines (IR-801, IR-802, and IR-803) were provided by Immune Response Inc. (San Diego, CA). T2 cell line and GBM cell lines, including U-373MG, U-118MG, U-138MG, and U-87MG, were supplied by American Type Culture Collection (Manassas, VA). The COS-7. A1 cell line was provided by the National Cancer Institute (Bethesda, MD). The cells were maintained in RPMI 1640 (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum (Irvine Scientific), 10 mM HEPES buffer, 100 units/ml penicillin-streptomycin (Invitrogen), 2 mM L-glutamine (Invitrogen), and 0.05 mM β-mercaptoethanol (Sigma, St. Louis, MO).

***In Vitro* Treatment of Tumor Cells with 5-Aza-2′-deoxycytidine (DAC).** Primary glioblastoma cells were seeded at a density of 3–4 $\times 10^5$ cells/ml in a T75 tissue culture flask. When cells became firmly adherent to plastic, the medium was replaced with fresh medium containing 1 μM DAC (Sigma) every 12 h for 3 days (six pulses). At the end of treatment, the medium was replaced with fresh culture medium without DAC for an additional 48 h and used for molecular and functional assays. Control cultures were treated under similar experimental conditions in the absence of DAC.

Synthetic Peptides and HLA Typing. All of the peptides using in this study were synthesized by Macromolecular Resource (Fort Collins, CO). The identity and purity of each of the peptides were confirmed by mass spectrometer and high-performance liquid chromatography analysis. Peptides were dissolved in DMSO at 1 mM concentration for future use. GBM cells were stained with biotin-conjugated HLA-A2- or HLA-A1-specific monoclonal antibody (US Biological, Swampscott, MA) or biotin-conjugated isotype control antibody. After streptavidin-PerCP (BD PharMingen, San Diego, CA) staining for 30 min, the mean fluorescence intensity of HLA-A2 staining was analyzed by flow cytometry.

RNA Isolation and cDNA Synthesis. Total cellular RNA was extracted from the primary glioblastoma cell lines (passage 3–4) using the RNA4PCR kit (Ambion, Austin, TX) according to the manufacturer's protocol. For cDNA synthesis, ~1 μg of total RNA was reverse transcribed into cDNA using random oligo(dT) and reverse transcriptase. cDNA was stored at -20°C for PCR.

Detection of the Expression of MAGE-1, gp100, and HER-2 mRNA by RT-PCR. The PCR mixture consisted of 5 μl of 10× thermophilic DNA polymerase reaction buffer, 4 μl of 10 mM deoxynucleotide triphosphate, 3 μl of $MgCl_2$ (25 mM), 100 pM each primer, 5 units of AmpliTag DNA polymerase, and 2 μl of reverse transcription mixture. DNase- and RNase-free water was added to bring the reaction volume to 50 μl. The PCR program was set as follows: 1 cycle of 95°C for 5 min; 35 cycles of 95°C for 30 s, 55°C for 1 min, and 72°C for 1 min; and a final extension at 72°C for 10 min. Primers for β-actin, gp100, and MAGE-1 used for this study were identical to those used in our previous report (32). HER-2 primers and product were as follows: forward, 5′-TCTGACGTCCATCGTCTCTG-3′; reverse, 5′-AGGGCATA-AGCTGTGTCACC-3′, and amplification product, 458 bp.

A 15-μl aliquot of each reaction was electrophoresed through a 1.5% agarose gel and stained with ethidium bromide for digital photograph under UV light, and the expected band size was assessed.

Flow Cytometry Analysis for Protein Expression. Immunostaining for HER-2, gp100, and MAGE-1 protein expression was performed using an intracellular staining kit (BD PharMingen) according to the manufacturer's recommendations. Appropriate dilutions of anti-HER-2, anti-Pmel (gp100) HMB-45 clone, anti-MAGE-1 monoclonal antibody from Lab Vision (Fremont, CA), and isotype control mouse IgG1 (BD PharMingen) were incubated with tumor cells for 45 min at 4°C in 2% heat-inactivated fetal bovine serum/PBS. Secondary staining consisted of FITC-conjugated goat antimouse IgG1 (DAKO, Carpinteria, CA). The relative expression of tumor antigen in the different cell lines was determined by fluorescence intensity ratio (r), which was obtained by dividing tumor antigen mean fluorescence intensity by individual isotype control mean fluorescence intensity (33). We set the following criteria for evaluating the levels of tumor antigens: $r < 1.6$ denotes no detectable protein expression; $1.6 \leq r < 3.0$ denotes weak expression; and $r \geq 3.0$ denotes strong expression.

Tumor Cell Recognition by CTL Assay. CTL cells were plated with target cells in 96-well round-bottomed plates in 200 μl of complete medium. After 18–24 h of incubation at 37°C, the supernatant was harvested for detection of IFN-γ release using ELISA kits (Endogen, Cambridge, MA). To determine whether cytotoxic activity was MHC I dependent, tumor cells were incubated with W6/32 (anti-HLA class I) monoclonal antibody from American Type Culture Collection at a final concentration of 50 μg/ml for 1 h at 37°C before the addition of CTLs. Coefficient of variation of intra- and interassay of ELISA assay is <10%.

Generation and Cloning of HER-2- and MAGE-1-Specific CTLs by *in Vitro* Stimulation with Peptides. Peripheral blood mononuclear cells (PBMCs) obtained from HLA-A2$^+$ or HLA-A1$^+$ GBM patients were prepared by Ficoll-Paque (Invitrogen) density gradient centrifugation. Cells were seeded (1×10^7 cells/3 ml/well) into 6-well plates (Costar, Cambridge, MA) in RPMI 1640 supplemented with 10% human AB blood phenotype serum, 2 mM L-glutamine, 10 mM HEPES, and antibiotics. After 2 h of incubation at 37°C, adherent cells were used for DC generation as described previously (32). Mature DCs were incubated with 10 μg/ml HER-2 or MAGE-1 peptide at 37°C for 2 h. Some 2×10^5 peptide-pulsed DCs were cultured with 2×10^6 autologous T cells in 2 ml of RPMI 1640 containing 5% human AB serum, IL-6 (1000 units/ml; R&D Systems, Minneapolis, MN), and IL-12 (5 ng/ml; R&D Systems) in 10 wells of 24-well culture plates (Falcon; Becton Dickinson, San Jose, CA). T-cell cultures were restimulated weekly with 2×10^5 peptide-pulsed DCs in the presence of 100 IU/ml IL-2 (Proleukin; Chiron, Emeryville, CA) and 5 ng/ml IL-7 (R&D Systems). Cultures were maintained at $<1.5 \times 10^6$ T cells/ml. Six days after the third stimulations, an aliquot of each T-cell culture was examined to evaluate the antigen-specific T cells by IFN-γ release. T cells from polyclonal cultures containing specific T cells were cloned by limiting dilution (34). Wells with the highest antigen-specific IFN-γ secretion were identified for further expansion.

Rapid Expansion of HER-2- and MAGE-1-Specific Clones. After specificity testing, 1–2 $\times 10^5$ specific T cells were resuspended in 50 ml of RPMI 1640 containing IL-2 (300 IU/ml), OKT3 (30 ng/ml), IL-7 (10 ng/ml), 10×10^6 irradiated allogeneic feeder cells from three randomly chosen individuals, and 5×10^6 irradiated autologous PBMCs pulsed with HER-2 or MAGE-1 peptide for 4 h at room temperature. The rationale for using the three pooled irradiated allogeneic feeder cells was that these may secrete additional cytokines that support the rapid expansion of CTLs. The cells were cultured in the smallest ledge of a 25-cm^2 flask for optimal density (45-degree angle). On day 10, the flask was placed upright; the cells were harvested or restimulated and given half-fresh medium with replenishment of cytokines. On day 14, cells were harvested and either prepared for additional expansion cycles or cryopreserved. In general, one cycle expansion of CTL clones resulted in a 50–100-fold increase in cell number.

Table 1 *Expression of HER-2, gp100, and MAGE-1 mRNA in GBM[a] cells and normal brain tissue*

Total RNA was isolated from 43 primary GBM cell lines (p3–4 level), 7 GBM tumor cell lines, and 6 normal brain tissue samples and reverse transcribed into cDNA. RT-PCR was performed using primers specific for HER-2, gp100, and MAGE-1 (see "Materials and Methods").

GBM cell line	HER-2	gp100	MAGE-1
U-87MG	+	+	+
U-118MG	+	−	+
U-373MG	−	−	+
U-138MG	−	+	+
IR-801	+	+	+
IR-802	+	−	+
IR-803	+	+	+
Normal brain tissue (n = 6)	66.7%[b]	50%[b]	0%[b]
Primary tumor cell lines (n = 43)	81.4%[b]	46.5%[b]	39.5%[b]

[a] GBM, glioblastoma multiforme; RT-PCR, reverse transcription-PCR.
[b] Data indicate the percentage of samples that had positive expression.

Tetramer Staining. HER-2-, gp100-, and MAGE-1-specific peptide tetramer (phycoerythrin-peptide loaded HLA tetramer complexes) were synthesized and provided by Beckman Coulter (San Diego, CA). Specific CTL clone CD8 cells were resuspended at 10^5 cells/50 μl fluorescence-activated cell-sorting buffer (phosphate buffer plus 1% inactivated fluorescence-activated cell-sorting buffer). Cells were incubated with 1 μl of tHLA for 30 min at room temperature, and incubation was then continued for 30 min at 4°C with 10 μl of anti-CD8 monoclonal antibody (Becton Dickinson). Cells were washed twice in 2 ml of cold fluorescence-activated cell-sorting buffer before analysis by fluorescence-activated cell sorting (Becton Dickinson).

Statistical Analysis. Spearman rank correlation coefficients (R) were evaluated to determine whether there was an association between antigen expression and CTL recognition determined by IFN-γ cytokine release assays. Paired t tests were used to determine the effects of IFN-γ and DAC tumor cell treatment on cytokine release by CTL clones. A difference of $P < 0.05$ was considered significant.

RESULTS

HER-2, gp100, and MAGE-1 mRNA Expression in Primary Cultured GBM Cells. Forty-three primary cultured GBM cell lines, seven GBM tumor cell lines, and six fresh normal brain tissues from trauma patients were used to examine the mRNA expression of HER-2, gp100, and MAGE-1 by RT-PCR. The distribution of tumor antigen mRNA expression in seven GBM cell lines is listed in Table 1. HER-2, gp100, and MAGE-1 mRNA expression was detected in 81.4%, 46.5%, and 39.5%, respectively, of the 43 GBM primary cell lines. In normal brain tissue, MAGE-1 was not detected, although HER-2 and gp100 were detected in 66.7% and 50% of the samples, respectively.

Expression of HER-2, gp100, and MAGE-1 Protein in GBM Tissue and Primary Cultured Tumor Cells. To determine whether HER-2, gp100, and MAGE-1 proteins were expressed within GBM cell lines, primary cultured cell lines were processed for immunofluorescence staining with specific monoclonal antibody and analyzed by flow cytometry. Based on the criteria of fluorescence intensity ratio (r; see "Materials and Methods"), HER-2, gp100, and MAGE-1 protein expression was found in 76%, 45%, and 38%, respectively, of the 43 primary tumor cell lines tested. Representative samples are shown in Fig. 1. Two independent experiments were performed on seven established GBM cell lines; data are shown in Table 2. We did not find protein expression in cell lines apparently lacking mRNA expression.

Characterization of MAGE-1, gp100, and Her-2 CTL Clones. To analyze whether HER-2-, gp100-, and MAGE-1-derived epitopes are presented by GBM tumor cells, we generated tumor antigen-specific CTL clones. Based on previous reports of HER-2, gp100, and MAGE-1, one dominant epitope was chosen for each antigen to generate CTL clones: HLA-A2-restricted HER-2 ($9_{369-377}$) peptide (KIFGSLAFL); HLA-A1-restricted MAGE-1 ($9_{161-169}$) antigen peptide (EADPTGHSY); and HLA-A2-restricted gp100 ($9_{209-217}$) peptide (ITDQVPFSV). The gp100 CTL clone CK3H6 derived from melanoma tumor-infiltrating lymphocyte culture was provided by Dr. Mark E. Dudley (National Cancer Institute, NIH). Its specificity and activity have been well studied and published previously (16, 35). We used PBMCs from GBM patients and autologous DCs pulsed with peptide to generate HER-2 and MAGE-1 CTL clones. Autologous DCs from a single HLA-A2$^+$ patient and a single HLA-A1$^+$ patient were used to generate HER-2- and MAGE-1-specific CTL clones, respectively. PBMCs were stimulated by autologous DCs pulsed with

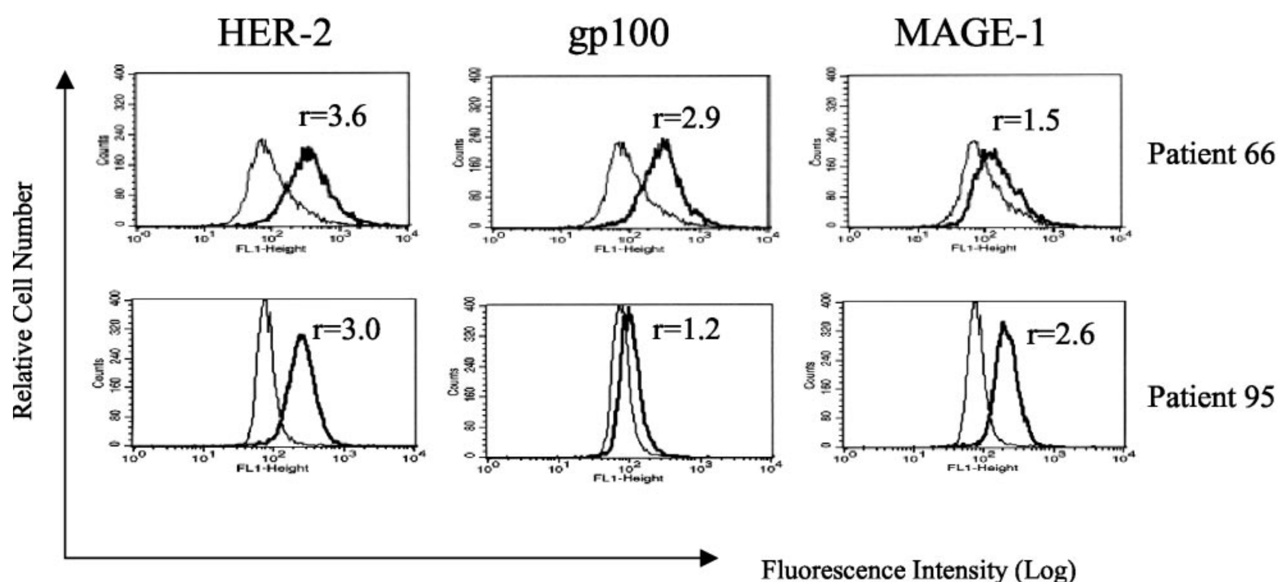

Fig. 1. Representative HER-2, MAGE-1 and gp100 protein expression in primary glioblastoma multiforme cell lines. The patient tumor cells at p3–4 level were collected and stained with specific monoclonal antibody to HER-2, MAGE-1, and gp100 and an isotype control-matched monoclonal antibody. Results are given as the ratio of fluorescence intensity (r). In the histograms, the *thick black line* represents staining with appropriate monoclonal antibody, and the *thin black line* represents the isotype control-matched monoclonal antibody (*i.e.,* background staining).

Table 2 *Expression of HER-2, gp100, and MAGE-1 protein in GBM[a] cell lines*

GBM tumor cell lines were cultured and stained with specific mAb to HER-2, MAGE-1, and gp100 and isotype control-matched mAb. The ratio of fluorescence intensity (r) in the table indicated antigen expression level (see "Materials and Methods").

	Experiment 1			Experiment 2		
GBM cell line	HER-2	gp100	MAGE-1	HER-2	gp100	MAGE-1
U-87MG	2.0	1.7	5.4	2.3	1.8	5.1
U-118MG	2.3	1.3	3.9	2.4	1.2	3.8
U-373MG	1.4	1.4	1.8	1.4	1.4	1.9
U-138MG	1.2	2.8	3.1	1.2	2.7	2.8
IR-801	3.2	2.6	2.0	3.4	2.5	1.9
IR-802	6.2	1.5	1.8	6.6	1.5	2.1
IR-803	2.6	2.7	2.6	2.3	2.5	2.4

[a] GBM, glioblastoma multiforme; mAb, monoclonal antibody.

peptide for three cycles, and then CTL was cloned by limited dilution. After testing the specificity and activity of growing CTLs, two of the highest peptide-specific IFN-γ-secreting clones, A05 for HER-2 and B07 for MAGE-1, were selected for further expansion. The specificity of each of the three CTL clones was verified by tetramer staining and cytokine release assay (Tables 3 and 4). Each CTL clone showed >93% positive staining for both CD8 and specific tetramer. No staining was seen with nonspecific tetramer (Fig. 2).

Recognition of Primary Cultured GBM Cell Line by HER-2-, gp100-, and MAGE-1-Specific CTL Clones. CTL clone cells (1×10^5) were incubated with 1×10^5 primary cultured GBM cells (p3–4 level) or GBM cells in 200 μl of complete medium. After incubation for 24 h, the recognition of the tumor cells by CTLs was assessed by the secretion of IFN-γ in supernatant. As shown in Tables 3 and 4, HER-2 CTL clone A05, gp100 clone CK3H6, and MAGE-1 clone B07 only recognized HLA-A2- or HLA-A1-matched GBM cells where specific tumor antigen expression was detected. The observation that these three CTL clones recognized tumor antigen in HLA class I restriction was further confirmed by HLA class I antibody (W6/32) blocking studies performed on one representative primary tumor cell line (No. 43). As shown in Tables 3 and 4, the levels of HER-2, gp100, and MAGE-1 protein expression correlated with T-cell release of INF-γ after coincubation with MHC-matching tumor cells ($r = 0.86$; $P < 0.01$). These data suggest that HER-2 ($9_{369-377}$), gp100 ($9_{209-217}$), and MAGE-1 ($9_{161-169}$) epitopes could be naturally processed and recognized by specific CTLs in a HLA class I-restricted manner.

Effects of IFN-γ Treatment on Recognition of GBM Cells by HER-2-, gp100-, and MAGE-1-Specific CTL Clones. IFN-γ treatment increased MHC class I expression on GBM cells (data not shown). The ratio of fluorescence intensity was increased by 5.2 ± 1.3-fold ($P < 0.05$). As shown in Tables 3 and 4, after IFN-γ treatment, the recognition of tumor cells by CTL clone A05, CK3H6, and B07 was significantly increased ($P < 0.05$).

DAC Treatment Regulates MAGE-1 Expression. We sought to determine the effects of DNA methylation on regulation of MAGE-1 expression and the recognition of GBM cells by MAGE-1-specific CTLs. RT-PCR of MAGE-1 mRNA and recognition by CTLs were monitored after exposure of GBM cells to 1 μM DAC for 72 h. As shown in Fig. 3, MAGE-1 transcript was up-regulated in primary GBM cell lines 148, 108, and 84 and induced by DAC in cell lines 44, 129, and 163.

As the results show in Tables 3 and 4, the levels of tumor antigen

Table 4 *Recognition of GBM[a] cells by MAGE-1-specific CTL clone B07*

CTL clone B07 cells (1×10^5) were incubated with 1×10^5 target cells for 24 h, and release of IFN-γ (pg/ml) was analyzed by ELISA.

			Target treatment	
GBM cell code	HLA-A1 expression	MAGE-1 protein expression[b]	Control	IFN-γ [c]
Unpulsed COS-7.A1	+		159	166
COS-7.A1+ MAGE-1 ($9_{161-169}$)	+	1 μM	**>2500**	**>2500**
79	+	++	**1499**	**1990**
4	+	++	**1238**	**1545**
94	+	++	**1102**	**1621**
169	+	++	**1259**	**2010**
43	+	++	**1108**	**1877**
43+W6/32[d]	+	++	218	235
147	+	+	**533**	**766**
84	+	+	**497**	**723**
129	+	−	245	315
163	+	−	187	215
108	+	−	196	234
44	+	−	148	176
22	+	−	175	198
U-118MG	−	++	165	154

[a] GBM, glioblastoma multiforme.
[b] MAGE-1 protein expression was stained and analyzed by flow cytometry. −, no detectable expression; +, weak expression; ++, strong expression.
[c] Tumor cells treated with 1000 pg/ml IFN-γ for 48 h.
[d] Tumor cells were incubated with W6/32 (anti-HLA class I) antibody at a final concentration of 50 μg/ml for 1 h at 37°C before the addition of CTLs. Results are representative of two individual experiments, and each value represents the mean from triplicate wells.

Table 3 *Recognition of primary cultured GBM[a] cells by HER-2 and gp100 CTL clone*

CTL clone cells (1×10^5) were incubated with 1×10^5 target cells for 24 h, and release of IFN-γ (in pg/ml) was analyzed by ELISA.

HER-2-specific CTL clone A05					gp100-specific CTL clone CK3H6				
			Target treatment					Target treatment	
GBM cell code	HLA-A2	HER-2[b]	Control	IFN-γ[c]	GBM cell code	HLA-A2	gp100[b]	Control	IFN-γ[c]
T2+gp100 ($9_{209-217}$)			116	122	T2+HER-2 ($9_{369-377}$)			106	112
T2+HER-2 ($9_{369-377}$)	+	1 μM	**>2500**	**>2500**	T2+gp100 ($9_{209-217}$)	+	1 μM	**>2500**	**>2500**
66	+	++	**1428**	**1950**	175	+	++	**1438**	**2297**
175	+	++	**1087**	**1783**	94	+	++	**1444**	**1986**
110	+	++	**857**	**1249**	296	+	++	**1314**	**1797**
43	+	++	**814**	**1278**	43	+	++	**921**	**1566**
43+w6/32[d]	+	++	117	155	43+w6/32[d]	+	++	121	148
296	+	++	**993**	**1345**	59	+	+	**810**	**1346**
22	+	+	**475**	**798**	22	+	+	**625**	**1037**
144	+	+	**477**	**745**	66	+	+	**501**	**851**
59	+	+	**466**	**816**	90	+	−	109	112
90	+	−	116	113	186	+	−	116	107
44	−	+	112	125	44	−	++	112	139
U-87MG	−	+	103	123	84	−	++	103	143

[a] GBM, glioblastoma multiforme.
[b] HER-2 and gp100 protein expression was stained and analyzed by flow cytometry. −, no detectable expression; +, weak expression; ++, strong expression.
[c] Tumor cells treated with 1000 pg/ml IFN-γ for 48 h.
[d] Tumor cells were incubated with W6/32 (anti-HLA class I) antibody at a final concentration of 50 μg/ml for 1 h at 37°C before the addition of CTLs. Results are representative of two individual experiments, and each value represents the mean from triplicate wells.

Fig. 2. Assessment of HER-2, gp100, and MAGE-1 CTL clone by HLA/peptide tetramer staining. HER-2-, gp100-, and MAGE-1-specific CTL clone A05, CK3H6, and B07 (1×10^5 cells) were stained with phycoerythrin-conjugated peptide/HLA tetramer at room temperature for 30 min. Cells were then incubated with antibody for CD8 for 30 min at 4°C. Cells were examined by fluorescence-activated cell-sorting analysis using 10,000 events/sample.

protein determine the sensitivity of target cells to recognition by CTLs. As shown in Fig. 4, after treatment with DAC, GBM could be efficiently recognized by HLA-A1-restricted MAGE-1-specific CTL clone B07, and IFN-γ release by B07 T cells was significantly increased compared with that of the control (without treatment; $P < 0.01$). DAC treatment generates sufficient amounts of immunogenic peptides to be loaded on preexisting MHC class I molecules, which provides cell surface copies of HLA class I complexes above the threshold level required for efficient CTL recognition of targets cells (36). Our data have shown that treatment of MAGE-1-negative GBM tumor cells with DAC induced MAGE-1 expression and increased their recognition by MAGE-1-specific CTLs.

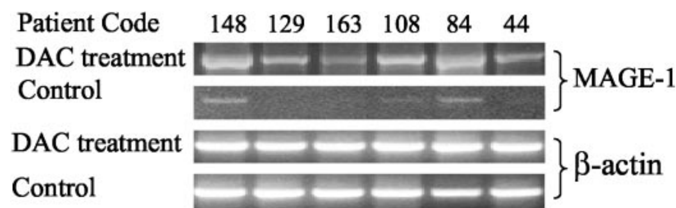

Fig. 3. Induction of MAGE-1 expression on primary glioblastoma multiforme tumor cell lines by 5-aza-2′-deoxycytidine (DAC). Paired cells were exposed to 1 μM DAC for 72 h (*DAC treatment*) or medium (*Control*). MAGE-1 mRNA expression was assessed by reverse transcription-PCR analysis after DAC treatment.

DISCUSSION

In this study, we demonstrated that HER-2, gp100, and MAGE-1 are expressed in human glioblastoma cells and also recognized by cytotoxic T cells. Antigen recognition is determined by both antigen expression and MHC expression on the cell surface. Our results demonstrated that tumor antigen expression in GBM cells correlates with tumor cell recognition by CTLs, which is in agreement with other reports on melanoma (11, 23). Our studies showed that IFN-γ treatment could significantly increase MHC class I expression on GBM tumor cells, further enhancing the sensitivity of recognition by CTLs. The demonstration of MHC up-regulation in human glioma cell lines leading to increased recognition by antigen-specific CTLs further supports the incorporation of IFN-γ in the effector phase of glioma immunotherapy.

MAGE-1 is a cancer/testis antigen, which is a novel family of immunogenic proteins. MAGE genes were initially analyzed from melanoma and shown to exhibit a nearly exclusive neoplasm-specific expression pattern. Our results showed that MAGE-1 was exclusively expressed in GBM tissues and cells and was not detected in normal brain. A study of childhood astrocytomas reported the immunohistochemical presence and cellular localization of the MAGE-1 antigen in all specimens of malignant gliomas including glioblastoma. No MAGE-1 expression was detected, however, in the lowest grade, pilocytic astrocytoma (4). Previous studies have defined the regulatory role of DNA methylation in the constitutive expression of cancer/

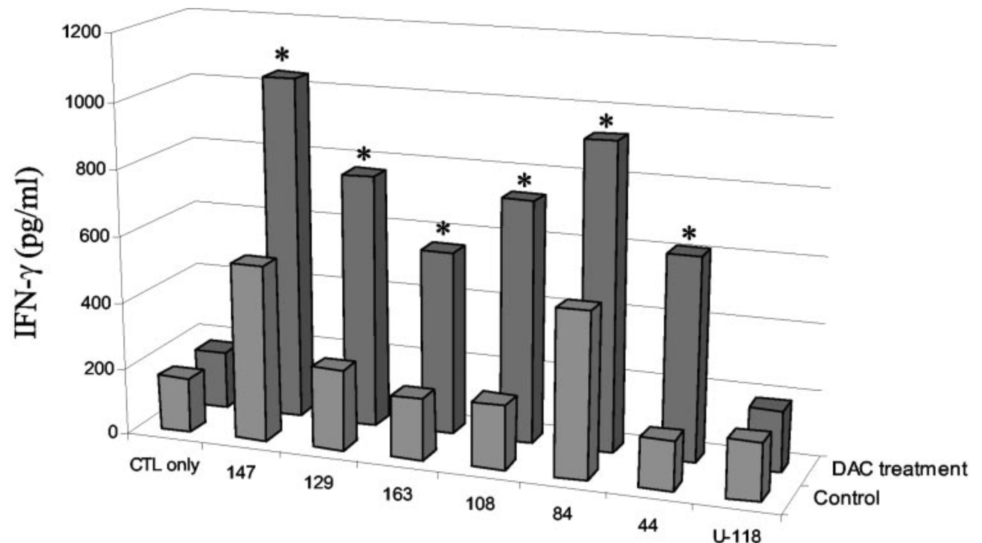

Fig. 4. Effects of 5-aza-2′-deoxycytidine (DAC) treatment on recognition of glioblastoma multiforme tumor cells by MAGE-1 CTL clone B07. Paired cells were exposed to 1 μM DAC for 72 h (*DAC treatment*) or medium (*Control*). IFN-γ release by CD8+ T cells was significantly increased when 1×10^5 B07 CTL cells were incubated with 1×10^5 DAC-treated tumor cells as compared with B07 CTL cells incubated with 1×10^5 control cells ($P < 0.01$). The *asterisks* indicate a significant increase of IFN-γ release by CTL clone incubated with DAC-treated glioblastoma multiforme cells over control ($P < 0.01$).

testis antigens by melanoma cells and renal cell carcinoma and showed that *in vitro* treatment with the DNA hypomethylating agent DAC induced and/or up-regulated functional cancer/testis antigen expression in neoplastic cells (36). In our studies, MAGE-1-negative GBM tumor cells, after exposure to 1 μM DAC for 72 h, demonstrated MAGE-1 mRNA expression by RT-PCR. Furthermore, DAC-treated GBM cells were recognized by the MAGE-1-specific MHC-restricted cytotoxic T-cell clone B07, whereas untreated control cells were not recognized. This finding suggests that production of the antigen encoded by the MAGE-1 gene was induced by DAC and that it was presented in association with MHC class I molecules at the cell surface for T-cell recognition. Normally, about 40% of GBM tumor cells are MAGE-1 positive, so it would be important to induce its expression before MAGE-1 immunization. Successful up-regulation of the MAGE-1 tumor antigen by DAC might be combined with a strategy to up-regulate MHC levels by IFN-γ to augment antigen recognition by T cells (37).

Rimoldi *et al.* (29) were the first to document that melanoma-associated antigen-specific CTL lines could recognize HLA-matched glioma cells *in vitro*. Then, Chi *et al.* (27), Scarcella *et al.* (28), and Sahin *et al.* (30) reported that gp100 and MAGE-1 mRNA was expressed in glioma tumor cells and tumor tissue by RT-PCR, and HER-2 was found in brain tumor by immunohistochemical staining (25, 26). More recently, Prins *et al.* (38) validated melanoma-associated antigen gp100 and TRP-2 as immunotherapeutic targets in a murine glioma model (38). Very importantly, IL-13 receptor α2 has been identified as a glioma-specific antigen (39), and a HLA-A2.1-restricted CTL epitope (WLPFGFILI) was defined by Okano *et al.* (40). Recently, we reported on TRP-2 as a CTL target in malignant glioma, which demonstrated that TRP-2 antigen can be naturally processed and recognized by TRP-2-specific CTLs. We also found that TRP-2-specific cytotoxic T-cell activity was detected when PBMCs were stimulated with autologous DCs pulsed with irradiated GBM tumor cells *in vitro* and in patents' PBMCs after DC-pulsed autologous tumor lysate vaccinations (41). The goal of tumor antigen identification study is to translate the finding from bench side to bedside. In our DC-based clinical trial, we found that vaccinations with DCs pulsed with tumor lysates were able to induce HER-2-, gp100-, and MAGE-1-specific CTLs in patients' PBMCs after vaccinations as determined by tetramer analysis.[3] Our demonstration of the presence of HER-2, gp100, and MAGE-1 expression on GBM cells and the generation of peptide-specific, MHC-restricted CTLs that recognized GBM cells lay the groundwork for the use of relevant surrogate assays to determine the generation of antigen-specific cytotoxicity.

Although the presence of gp100 and HER-2 in normal brain could be a concern, previous melanoma clinical trials with gp100 peptide and breast cancer clinical trials with HER-2 peptide did not elicit autoimmune responses, despite the generation of CD8+ and CD4+ T cells responsive to gp100 and HER-2. In adults, the HER-2 gene is present as a single copy in normal cells; however, amplification of the gene and resultant protein overexpression are seen in various cancers. It is possible that the epitopes expressed on normal tissues are below the threshold level for T-cell recognition, whereas their overexpression in tumor cells can trigger an anticancer response (10). Even if normal brain tissue expresses HER-2 and gp100 mRNA, most neural cells do not express MHC, preventing CTL recognition (42). In the past decade, HER-2, gp100, and MAGE-1 recombinant protein; recombinant viral vectors; DNA vaccines; and more class I and class II epitopes have been identified (43–46). The availability of such reagents highlight HER-2, gp100, and MAGE-1 as attractive brain tumor therapy target candidates. Our studies demonstrate that these tumor antigens can be effective targets for both CD8 and CD4 T-cell adoptive transfer and active specific immunotherapy in malignant glioma.

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REFERENCES

1. Black KL, Pikul BK. Gliomas: past, present, and future. Clin Neurosurg 1999;45: 160–3.
2. Surawicz TS, Davis F, Freels S, Laws ER Jr, Menck HR. Brain tumor survival: results from the National Cancer Data Base. J Neurooncol 1998;40:151–60.
3. Rosenberg SA, Packard BS, Aebersold PM, et al. Use of tumor-infiltrating lymphocytes and interleukin-2 in the immunotherapy of patients with metastatic melanoma. A preliminary report. N Engl J Med 1988;319:1676–80.
4. van der Bruggen P, Traversari C, Chomez P, et al. A gene encoding an antigen recognized by cytolytic T lymphocytes on a human melanoma. Science (Wash DC) 1991;254:1643–7.
5. Traversari C, van der Bruggen P, Luescher IF, et al. A nonapeptide encoded by human gene MAGE-1 is recognized on HLA-A1 by cytolytic T lymphocytes directed against tumor antigen MZ2-E. J Exp Med 1992;176:1453–7.
6. Inoue H, Mori M, Li J, et al. Human esophageal carcinomas frequently express the tumor-rejection antigens of MAGE genes. Int J Cancer 1995;63:523–6.

[3] Yu JS, Liu G, Ying H, et al. Vaccination with tumor lysate-pulsed dendritic cells elicits antigen specific cytotoxic T cells in patients with malignant glioma, in press.

7. Gotoh K, Yatabe Y, Sugiura T, et al. Frequency of MAGE-3 gene expression in HLA-A2 positive patients with non-small cell lung cancer. Lung Cancer 1998;20:117–25.
8. Chen CH, Huang GT, Lee HS, et al. High frequency of expression of MAGE genes in human hepatocellular carcinoma. Liver 1999;19:110–4.
9. Yamanaka K, Miyake H, Hara I, et al. Expression of MAGE genes in renal cell carcinoma. Int J Mol Med 1998;2:57–60.
10. Renkvist N, Castelli C, Robbins PF. A list of human tumor antigens recognized by T cells. http://www.cancerimmunity.org/statics/database.htm, 2003.
11. Riker AI, Kammula US, Panelli MC, et al. Threshold levels of gene expression of the melanoma antigen gp100 correlate with tumor cell recognition by cytotoxic T lymphocytes. Int J Cancer 2000;86:818–26.
12. Lee RS, Tartour E, van der Bruggen P, et al. Major histocompatibility complex class I presentation of exogenous soluble tumor antigen fused to the B-fragment of Shiga toxin. Eur J Immunol 1998;28:2726–37.
13. Kawakami Y, Eliyahu S, Delgado CH, et al. Identification of a human melanoma antigen recognized by tumor-infiltrating lymphocytes associated with in vivo tumor rejection. Proc Natl Acad Sci USA 1994;91:6458–62.
14. Okamoto T, Irie RF, Fujii S, et al. Anti-tyrosinase-related protein-2 immune response in vitiligo patients and melanoma patients receiving active-specific immunotherapy. J Investig Dermatol 1998;111:1034–9.
15. Kawakami Y, Eliyahu S, Jennings C, et al. Recognition of multiple epitopes in the human melanoma antigen gp100 by tumor-infiltrating T lymphocytes associated with in vivo tumor regression. J Immunol 1995;154:3961–8.
16. Dudley ME, Wunderlich JR, Robbins PF, et al. Cancer regression and autoimmunity in patients after clonal repopulation with antitumor lymphocytes. Science (Wash DC) 2002;298:850–4.
17. Coussens L, Yang-Feng TL, Liao YC, et al. Tyrosine kinase receptor with extensive homology to EGF receptor shares chromosomal location with neu oncogene. Science (Wash DC) 1985;230:1132–9.
18. Slamon DJ, Godolphin W, Jones LA, et al. Studies of the HER-2/neu proto-oncogene in human breast and ovarian cancer. Science (Wash DC) 1989;244:707–12.
19. Brossart P, Stuhler G, Flad T, et al. Her-2/neu-derived peptides are tumor-associated antigens expressed by human renal cell and colon carcinoma lines and are recognized by in vitro induced specific cytotoxic T lymphocytes. Cancer Res 1998;58:732–6.
20. Seliger B, Rongcun Y, Atkins D, et al. HER-2/neu is expressed in human renal cell carcinoma at heterogeneous levels independently of tumor grading and staging and can be recognized by HLA-A2.1-restricted cytotoxic T lymphocytes. Int J Cancer 2000;87:349–59.
21. Bernhard H, Salazar L, Schiffman K, et al. Vaccination against the HER-2/neu oncogenic protein. Endocr Relat Cancer 2002;9:33–44.
22. Lustgarten J, Theobald M, Labadie C, et al. Identification of Her-2/Neu CTL epitopes using double transgenic mice expressing HLA-A2.1 and human CD. 8. Hum Immunol 1997;52:109–18.
23. Fisk B, Blevins TL, Wharton JT, Ioannides CG. Identification of an immunodominant peptide of HER-2/neu protooncogene recognized by ovarian tumor-specific cytotoxic T lymphocyte lines. J Exp Med 1995;181:2109–17.
24. Gilbertson RJ, Pearson AD, Perry RH, Jaros E, Kelly PJ. Prognostic significance of the c-erbB-2 oncogene product in childhood medulloblastoma. Br J Cancer 1995;71:473–7.
25. Schwechheimer K, Laufle RM, Schmahl W, et al. Expression of neu/c-erbB-2 in human brain tumors. Hum Pathol 1994;25:772–80.
26. Hiesiger EM, Hayes RL, Pierz DM, Budzilovich GN. Prognostic relevance of epidermal growth factor receptor (EGF-R) and c-neu/erbB2 expression in glioblastomas (GBMs). J Neurooncol 1993;16:93–104.
27. Chi DD, Merchant RE, Rand R, et al. Molecular detection of tumor-associated antigens shared by human cutaneous melanomas and gliomas. Am J Pathol 1997;150:2143–52.
28. Scarcella DL, Chow CW, Gonzales MF, et al. Expression of MAGE and GAGE in high-grade brain tumors: a potential target for specific immunotherapy and diagnostic markers. Clin Cancer Res 1999;5:335–41.
29. Rimoldi D, Romero P, Carrel S. The human melanoma antigen-encoding gene, MAGE-1, is expressed by other tumour cells of neuroectodermal origin such as glioblastomas and neuroblastomas. Int J Cancer 1993;54:527–8.
30. Sahin U, Koslowski M, Tureci O, et al. Expression of cancer testis genes in human brain tumors. Clin Cancer Res 2000;6:3916–22.
31. Heim S. Cancer cytogenetics: chromosomal and molecular genetic aberrations of tumor cells, 2nd ed. New York: Wiley-Liss, Inc.; 1995.
32. Yu JS, Wheeler CJ, Zeltzer PM, et al. Vaccination of malignant glioma patients with peptide-pulsed dendritic cells elicits systemic cytotoxicity and intracranial T-cell infiltration. Cancer Res 2001;61:842–7.
33. Pellat-Deceunynck C, Mellerin MP, Labarriere N, et al. The cancer germ-line genes MAGE-1, MAGE-3 and PRAME are commonly expressed by human myeloma cells. Eur J Immunol 2000;30:803–9.
34. Fonteneau JF, Larsson M, Somersan S, et al. Generation of high quantities of viral and tumor-specific human CD4+ and CD8+ T-cell clones using peptide pulsed mature dendritic cells. J Immunol Methods 2001;258:111–26.
35. Dudley ME, Wunderlich J, Nishimura MI, et al. Adoptive transfer of cloned melanoma-reactive T lymphocytes for the treatment of patients with metastatic melanoma. J Immunother 2001;24:363–73.
36. De Smet C, De Backer O, Faraoni I, et al. The activation of human gene MAGE-1 in tumor cells is correlated with genome-wide demethylation. Proc Natl Acad Sci USA 1996;93:7149–53.
37. Weber J, Salgaller M, Samid D, et al. Expression of the MAGE-1 tumor antigen is up-regulated by the demethylating agent 5-aza-2′-deoxycytidine. Cancer Res 1994;54:1766–71.
38. Prins RM, Odesa SK, Liau LM. Immunotherapeutic targeting of shared melanoma-associated antigens in a murine glioma model. Cancer Res 2003;63:8487–91.
39. Debinski W, Gibo DM, Hulet SW, Connor JR, Gillespie GY. Receptor for interleukin 13 is a marker and therapeutic target for human high-grade gliomas. Clin Cancer Res 1999;5:985–90.
40. Okano F, Storkus WJ, Chambers WH, Pollack IF, Okada H. Identification of a novel HLA-A*0201-restricted, cytotoxic T lymphocyte epitope in a human glioma-associated antigen, interleukin 13 receptor alpha2 chain. Clin Cancer Res 2002;8:2851–5.
41. Liu G, Khong HT, Wheeler CJ, et al. Molecular and functional analysis of tyrosinase-related protein (TRP)-2 as a cytotoxic T lymphocyte target in patients with malignant glioma. J Immunother 2003;26:301–12.
42. Kruse CA, Cepeda L, Owens B, et al. Treatment of recurrent glioma with intracavitary alloreactive cytotoxic T lymphocytes and interleukin-2. Cancer Immunol Immunother 1997;45:77–87.
43. Sotiriadou R, Perez SA, Gritzapis AD, et al. Peptide HER2(776-788) represents a naturally processed broad MHC class II-restricted T cell epitope. Br J Cancer 2001;85:1527–34.
44. Bonini C, Lee SP, Riddell SR, Greenberg PD. Targeting antigen in mature dendritic cells for simultaneous stimulation of CD4+ and CD8+ T cells. J Immunol 2001;166:5250–7.
45. Kobayashi H, Lu J, Celis E. Identification of helper T-cell epitopes that encompass or lie proximal to cytotoxic T-cell epitopes in the gp100 melanoma tumor antigen. Cancer Res 2001;61:7577–84.
46. Lapointe R, Royal RE, Reeves ME, et al. Retrovirally transduced human dendritic cells can generate T cells recognizing multiple MHC class I and class II epitopes from the melanoma antigen glycoprotein 100. J Immunol 2001;167:4758–64.