Immunotheapeutic Targeting of Shared Melanoma-Associated Antigens in a Murine Glioma Model

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ABSTRACT

Immune-based treatments for central nervous system gliomas have traditionally lagged behind those of more immunogenic tumors such as melanoma. The relative paucity of defined glioma-associated antigens that can be targeted by the immune system may partially account for this situation. Antigens present on melanomas have been extensively characterized, both in humans and in murine preclinical models. Melanocytes and astrocytes are both derived embryologically from the neural ectoderm. Their neoplastic counterparts, malignant melanomas and gliomas, have been shown in humans to share common antigens at the RNA level. However, little is known concerning whether gliomas can be targeted by immune-based strategies that prime T cells to epitopes from melanoma-associated antigens (MAAs). In this study, we provide evidence that two common murine glioma cell lines (GL26 and GL261) express the melanoma antigens gp100 and tyrosinase-related protein 2 (TRP-2). To understand the immunogenicity of murine gliomas to CD8+ T cells, we examined the ability of a MAA-specific CTL cell line to lyse the glioma cells, as well as the in vivo expansion of MAA-specific CD8+ T cells in animals harboring gliomas. Both glioma cell lines were lysed by a human gp100-specific CTL cell line in vitro. Mice harboring s.c. GL26 gliomas possessed TRP-2-specific CD8+ T cells, providing further evidence that these gliomas express the protein products in the context of MHC class I. Furthermore, MAA peptide-pulsed dendritic cells could prime T cells that specifically recognize GL26 glioma cells in vitro. Lastly, mice that were prevaccinated with human gp100 and TRP-2 peptide-pulsed dendritic cells had significantly extended survival when challenged with tumor cells in the brain, resulting in >50% long-term survival. These results suggest that shared MAAs on gliomas can be targeted immunotheapeutically, pointing the way to a new potential treatment option for patients with malignant gliomas.

INTRODUCTION

Malignant gliomas represent a significant class of CNS tumors derived from the glial lineage. Despite recent advances in traditional treatment options, the prognosis for these patients has not changed appreciably. The 5-year survival rate for patients harboring the most common class of gliomas, glioblastoma multiforme, is less than 2% (1). In an effort to improve the outcome of patients with resectable brain tumors, there have been attempts to give adjunctive therapies consisting of radiation with or without chemotherapy. Thus far, research over three decades has failed to provide definitive evidence of improved outcome (i.e., overall survival and disease-free survival) in patients. Clearly, modalities other than radiation and/or chemotherapy must be explored in an adjuvant setting.

Immune-based treatments represent a promising new class of therapy designed to harness the immune system to specifically eradicate malignant cells. However, immunotherapy for tumors located in the CNS has generally not achieved the results seen for peripherally located tumors (2–5). These previous findings have historically been ascribed to the “immune privilege” of the CNS (6). In reality, however, the immune privilege of the brain is not absolute but was originally used to describe the observation that tissue and tumor grafts survived better in the CNS than in other peripheral sites (7). Effective anti-CNS tumor immune responses have been generated by immune-based treatments such as adoptive T-cell transfer (8–13), GAA-pulsed DCs (14–17), and cytokine-secreting gliomas or fibroblasts (2, 3, 18–20), lending further credence to the idea that the efficient induction of a cellular antitumor immune response can be targeted to antigens within the CNS.

The previous preclinical studies, however, have been hampered by the absence of a defined CTL recognizable antigen naturally expressed on gliomas. In this study we have explored the utility of targeting shared MAAs expressed on the murine GL26 glioma. We found that the GL26 glioma expresses at least two of the well-characterized murine MAAs, gp100 and TRP-2. CTL epitopes have been extensively characterized for these two melanoma antigens (21–23). This study confirms that the murine GL26 glioma can be targeted using MAA-pulsed DCs in an immunologically relevant fashion.

MATERIALS AND METHODS

Animals and Cell Lines. Female C57BL/6 (H-2b) mice, 6–10 weeks of age, were obtained from Jackson Laboratories (Bar Harbor, ME). All animals were maintained in a barrier facility, and all experiments were approved by the University of California Los Angeles Animal Research Committee. The murine glioma cell lines were obtained from Drew Pardoll (Johns Hopkins University, Baltimore, MD) and grown in DMEM:Ham’s F-12 + 10% fetal bovine serum and 1% penicillin/streptomycin (TM medium). The DC2.4 DC line was obtained from Ken Rock (University of Massachusetts, Worcester, MA) and grown in RPMI 1640 +10% fetal bovine serum, 1% penicillin/streptomycin, 2-mercapto ethanol, sodium pyruvate, and nonessential amino acids (lymphocyte medium). The B16-F10 melanoma was purchased from American Type Culture Collection (Manassas, VA) and grown in TM medium. Pmel-1 CD8+ T cells were obtained from Nick Restifo (National Cancer Institute, NIH, Bethesda, MD). The hgp10025–35-specific Pmel-1 TCR transgenic T cells were generated from mice expressing αβTCR CDNA from a hgp10025–35-specific, H-2Db-restricted CD8+ T-cell clone (clone 9), as described previously (22, 24). The cells were grown in lymphocyte medium.

JAM Cytotoxicity Assays. TCR transgenic Pmel-1 T cells were restimulated with 1 μg hgp10025–35 peptide-pulsed and irradiated splenocytes for 5 days with 30 units/ml human IL-2. Tumor cells were incubated with 5 μCi/ml [3H]thymidine for 24 h and then put into 96-well plates at the indicated ratios with the effector cells for 6 h at 37°C, 5% CO2 in a JAM cytotoxicity assay as described previously (25). [3H]thymidine incorporation was measured using a β plate reader.

Cytokine Release Assays. Splenocyte single cell suspensions were prepared from treated mice and cocultured with irradiated tumor cells as described previously (3). Briefly, splenocytes were erythrocyte depleted with a Tris-ammonium chloride solution (PharmLyse; Pharmingen, San Diego, CA) and

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The abbreviations used are: CNS, central nervous system; i.c., intracranial; TRP-2, tyrosinase-related protein 2; MAA, melanoma-associated antigen; GAA, glioma-associated antigen; DC, dendritic cell; TCR, T-cell receptor; hgp100, human gp100; IL, interleukin; mgi100, murine gp100; PBS, phosphate buffered saline.
cultured in 6-well plates at a 50:1 E:T ratio with irradiated tumor cells for 5 days. Recombinant human IL-2 (30 units/ml) was supplemented in the cultures. After 5 days of culture, the cell-free supernatants were tested for the presence of IFN-γ by ELISA (PharMingen).

Fluorescence-Activated Cell-Sorting Analysis and Antibodies. Splenocytes were stained with a multicolor monoclonal antibody mixture for lymphocyte subpopulations, activation markers and specific tetramers to H-2D(b)/hgp100\textsubscript{25–33} and H-2K(b)/TRP-2\textsubscript{180–188}, and control tetramer to listeriolysin, H-2K(b)/bLLO. Antibodies to CD8α (clone 53-6.7), CD4 (clone RM4-5), CD3ε (clone 145-2C11), TCRβ (clone H57-597), and IFN-γ (clone XMG1.2) were all purchased from BD PharMingen (San Diego, CA). Tetramers were obtained from the National Institute of Allergy and Infectious Diseases MHC tetramer core facility (Atlanta, GA). Cells were acquired on a FACSCalibur machine (BD Pharmingen) using CellQuest software and analyzed with FCS Express V2.

DC Pulsing and Vaccination. The DC2.4 cells were washed in PBS and pulsed with 10 μg hgp100\textsubscript{25–33} (KVPVRNQDWL) and/or TRP-2\textsubscript{180–188} (SVDFFVWL) peptides for 1.5 h at 37°C. The cells were then growth arrested with mitomycin C for 30 min, followed by extensive washing in PBS. The peptide-pulsed DC2.4 cells (1 x 10\textsuperscript{7} cells/animal) were injected intradermally on the flank.

Reverse Transcription-PCR for Melanoma Antigen Transcripts. Total RNA was isolated from cultured GL26, GL261, and B16-F10 cells using the RNAqueous 4PCR RNA kit (Ambion Inc., Austin, TX). For cDNA synthesis, approximately 1 μg of total RNA was reverse-transcribed using the Omniscript reverse transcriptase kit (Qiagen). The PCR mixture consisted of 300 μM deoxynucleotide triphosphates, 1.5 mM Mg\textsuperscript{2+}, 1 mM primers, and 2.5 units of polymerase/reaction. Primers for PCR were as follows: (a) mgp100, 5′-ATGGTGGGTGTCCAGAGAAG-3′ (forward) and 5′-GCACTCTTCCCCAGCACTT-3′ (reverse), product size = 192 bp; and (b) TRP-2, 5′-CAGACGGCATCTCAGTTTCCGCCAGCTCTG-3′ (forward) and 5′-CTCTGACACTTAAATTGGCCGGCAACAGAATAA-3′ (reverse), product size = 546 bp. The expression of gp100 and TRP-2 was confirmed by bands at the indicated product sizes on ethidium bromide-stained agarose gels.

Tumor Implantation. For s.c. glioma models, animals were anesthetized with a ketamine/xylazine mixture and then shaved. GL26 cells (1 x 10\textsuperscript{7}) were implanted on the flank. For the i.c. implantation of GL26 glioma cells, animals were anesthetized with ketamine/xylazine. The head was shaved, and the skull was exposed. Thereafter, the animal was positioned into a stereotactic frame. The head was shaved, and the skull was exposed. Thereafter, the animal was positioned into a stereotactic frame (David Kopf) with small animal earbars. A burr hole was made using a Dremel drill approximately 3 mm lateral and 1 mm posterior from the intersection of the coronal and sagittal sutures (bregma). Cells (1 x 10\textsuperscript{7}) were injected using a Hamilton syringe at a depth of 3 mm in a volume of 2 μl.

Statistical Analysis. Data were analyzed using the Microsoft Excel statistical package and graphed using Sigma Plot. Data are represented as the mean ± SE. Survival data were compared with the Wilcoxon and log-rank tests using the SAS statistical program. Student’s t tests were used to calculate the significance of all other data. Statistical significance was determined at the level of P < 0.05.

RESULTS

gp100 and TRP-2 Are Expressed by Murine Glioma Cells. Previous studies have demonstrated the shared expression of common melanoma antigens on human glioma cells (26, 27). However, data from preclinical models that can be used to ask more basic questions concerning specific immune responses directed to these antigens within the CNS are lacking. We isolated total RNA from the murine glioma cell lines GL26 and GL261 and tested for the expression of known melanoma antigens such as gp100 and TRP-2, which have well-characterized CTL epitopes. As shown in Fig. 1, we could detect the expression of both mgp100 and TRP-2 from both the GL26 and GL261 glioma cell lines. We also found that a gp100-specific CTL cell line would specifically lyse both GL26 and GL261 glioma cells, as well as the B16-F10 melanoma (Fig. 2). The relatively lower cytotoxicity found with B16-F10 is most likely attributable to the very low levels of MHC class I expression on this cell line because the pretreatment with IFN-γ dramatically increased MHC expression, as well as the percentage of lysed cells [data not shown and (28)]. These findings recapitulated which has been demonstrated previously in human studies (27, 29) but has not been shown in murine gliomas. These findings also provide a foundation to further test more clinically relevant questions concerning the ability to target MAAs immunotherapeutically in preclinical models. The remainder of the experimental studies were performed using the GL26 glioma.

TRP-2-Specific CD8\textsuperscript{+} T Cells Are Found in Mice Harboring Gliomas. To further test whether the murine antigens gp100 and TRP-2 are immunologically relevant, we inoculated mice with s.c. GL26 gliomas (1 x 10\textsuperscript{6} cells). For this experiment, the s.c. route was used for challenge with GL26 tumors because of the well-established growth conditions and easy detection of tumor development at the s.c. site. We tested for the presence of gp100 and TRP-2-specific CD8\textsuperscript{+} T cells using tetramers. We could not detect gp100-specific CTLs in glioma-bearing mice (data not shown). However, TRP-2-specific CD8\textsuperscript{+} T cells were readily detectable in the same glioma-bearing mice (Fig. 3; Table 1). These data strongly suggest that TRP-2 represents an immunogenic antigen that the immune system can and does recognize during glioma growth.

gp100\textsubscript{25–33} and TRP-2\textsubscript{180–188}-Primed T Cells Specifically Recognize Murine Glioma Cells. To test whether we could induce a specific antiglioma immune response using MAA as the immunogen, we pulsed DCs with either hgp100\textsubscript{25–33} or TRP-2\textsubscript{180–188} peptides and then vaccinated mice with two biweekly s.c. injections. We then...
Finally, to test whether MAA peptide-pulsed DCs could generate a clinically relevant antitumor immune response in vivo, we vaccinated animals with hgp100 and TRP-2 peptide-pulsed DCs and then challenged the animals with GL26 glioma cells in the brain 1 week later. Fig. 5 depicts a standard Kaplan-Meier plot that compares the survival between control groups and animals that received MAA peptide-pulsed DCs. There appeared to be a survival advantage conferred to mice that received vaccination with unpulsed DCs when compared with the untreated animals (data not shown), suggesting the possible effect of FCS antigens presented on unpulsed DCs alone. However, even after taking this background immunity into account, there was significant extension of survival for animals that received hgp100 and TRP-2 peptide-pulsed DCs compared with animals that received unpulsed DCs or hgp100 peptide-pulsed DCs (P < 0.02). These results suggest that the immune response induced by TRP-2 vaccination is capable of preventing glioma growth and extending survival under non-immunocompromised conditions.

**Table 1** Glioma-bearing animals possess significant numbers of TRP2-specific CD8+ T cells

<table>
<thead>
<tr>
<th>Group</th>
<th>%CD8+TRP-2+ cells</th>
<th>No. of CD8+TRP-2+ cells/spleen</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>1.25 ± 0.60</td>
<td>7.88 × 10^5 ± 4 × 10^5</td>
</tr>
<tr>
<td>GL26 tumor-bearing</td>
<td>3.83 ± 0.66*</td>
<td>3.38 × 10^6 ± 9 × 10^6*</td>
</tr>
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coclurated their splenocytes with irradiated tumor cells and tested for the secretion of IFN-γ by ELISA. We chose an altered peptide ligand for the gp100 epitope that has been shown to bind H-2D^d with greater affinity than the native sequence and could prime gp100-specific CTL responses (22). The TRP-2_180–198 Sequence has previously been well characterized as the most immunogenic epitope expressed in the TRP-2 amino acid sequence (21, 30). We first verified that the vaccination paradigm induced hgp100- and TRP-2-specific lymphocytes by IFN-γ ELISA and intracellular IFN-γ accumulation in primed animals (data not shown). Then we tested whether the same vaccination paradigm could prime lymphocytes to MAAs expressed on glioma cells in vitro. After vaccination with hgp100 or TRP-2 peptide-pulsed DCs, splenocytes from vaccinated animals were cocultured with B16, GL26, or spleen cells alone. MAA-primed splenocytes cultured without stimulator cells did exhibit some increased IFN-γ secretion over naïve splenocytes, which could be the result of an immune response to FCS antigens induced by vaccinating with DCs grown in FCS (31) or from residual T lymphocyte activation after the second DC vaccination 2 weeks earlier. Despite the possibility of a partial immune response due to the presence of FCS-induced immunity, however, both hgp100 and TRP-2 peptide-pulsed DCs primed a MAA-specific antitumor response that recognized glioma cells above and beyond the background reaction attributable to FCS alone (Fig. 4). These results suggest that the GL26 glioma cells express MAAs in the context of MHC that can be recognized by MAA-specific T cells.

MAA Peptide-Pulsed DC Vaccination Confers Antitumor Immune Protection to Subsequent i.c. GL26 Glioma Challenge. Finally, to test whether MAA peptide-pulsed DCs could generate a clinically relevant antitumor immune response in vivo, we vaccinated...
with the unrelated EL4 tumor cells (Fig. 6). Thus, the MAA peptide-pulsed DC vaccination resulted in tumor-specific immune memory.

**DISCUSSION**

In this study, we tested whether shared MAAs expressed on murine gliomas could be recognized and targeted immunologically in a preclinical brain tumor model. We showed that two common glioma cell lines, GL26 and GL261, express both gp100 and TRP-2. The expression of these MAAs on GL26 cells could be targeted by lymphocytes primed with hpgp100 and TRP-2 peptide-pulsed DC vaccination. In addition, animals harboring progressively growing gliomas also possessed large frequencies of TRP-2-specific CD8\(^+\) T cells. These data suggested that GL26 glioma-expressed TRP-2 was a naturally occurring immunogen recognized by endogenous CD8\(^+\) T cells, whereas GL26-expressed gp100 immunity could only be induced after hpgp100 peptide-pulsed DC vaccination. Finally, we showed that MAA peptide-pulsed DCs could induce protective immunity against GL26 gliomas implanted in the brain. Our finding of shared MAAs on murine gliomas provides for a unique opportunity to conduct further studies that ask basic questions regarding the requirements for targeting defined antigens located in organs that are normally "immunologically quiescent" (32). These results also provide a new experimental methodology to test clinically relevant questions in preclinical animal models.

The findings in this paper also add to a progressively growing list of antigens expressed on gliomas that can be targeted by the immune system. Rimoldi et al. (27) were the first to document that MAA-specific CTL lines could recognize HLA-matched glioma cells *in vitro*. More recently, Liu et al. (29) demonstrated additional evidence that TRP-2 could be immunologically relevant in human gliomas. They found that the frequency of TRP-2-specific CTLs was increased in patients with malignant gliomas after tumor lysate-pulsed DC vaccination (29). Other antigens with a more tumor-specific expression have also been targeted as well. Recently, a HLA-A2.1-restricted CTL epitope has been defined for the human IL-13 receptor \(\alpha\)2 chain (33), which is selectively overexpressed in human gliomas (34). Others are targeting glioma-associated proteins such as the transferrin receptor, epidermal growth factor receptor variants, and/or isoforms of tenasin using radiolabeled or immunotoxin-conjugated antibodies (35–38). Whereas some of these studies using passive immunotherapy have had encouraging results clinically, active immunotherapeutic approaches may provide an alternative or complementary approach to activate the host immune system to GAAs. Endogenous, GAA-primed lymphocytes can seek out and target tumor cells found within the CNS. This approach could be especially effective for isolated, small pockets of tumor cells that frequently contribute to tumor progression.

Although gp100 and TRP-2 are both nonmutated self-antigens, we did not see any evidence of autoimmunity after MAA peptide-pulsed DC vaccination in our murine glioma model. In previous studies, we also did not find any autoimmune sequelae when 9L glioma peptide-pulsed DCs were used to vaccinate rats (16). Thus, we believe that the induction of self-reactive MAA-specific T cells may be safe and that additional preclinical studies of efficacy are warranted. Conceivably, such T-cell-based immunotherapeutic strategies directed at well-defined MAAs might be used in the preclinical and clinical settings for the future treatment of gliomas. In preclinical studies, the adoptive transfer of MAA-specific T lymphocytes, alone or together with MAA-pulsed DCs, could be used to model the treatment of established or transgenic animal models of i.c. gliomas. Clinically, biopsy/ resection specimens from patients with gliomas can be easily screened for the presence of MAAs to determine appropriate candidates for immunotherapeutic approaches targeted at these antigens. Furthermore, because the immunogenic peptide epitopes of these MAAs are well defined and can be synthetically produced in large quantities, strategies targeting MAAs would bypass the need to acquire and process autologous glioma material as a source of antigen, which can often be a difficult and labor-intensive undertaking. Thus, our findings also suggest that immunotherapeutic approaches targeting defined human HLA-restricted MAA epitopes could be tested in the clinic for patients harboring CNS gliomas.

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