Glioblastoma Patients Exhibit Circulating Tumor-Specific CD8+ T Cells

Jie Tang,1 Phyllis Flomenberg,2,4 Larry Harshyne,1 Lawrence Kenyon,3 and David W. Andrews1, 4

Abstract

Purpose: There is growing interest in developing cellular immune therapies for glioblastoma multiforme, but little is known about tumor-specific T-cell responses. A glioblastoma multiforme–specific T-cell assay was developed using monocyte-derived dendritic cells to present tumor antigens from the established glioblastoma multiforme cell line U118.

Experimental Design: Peripheral blood mononuclear cells (PBMC) and tumor cells were obtained from nine patients with newly diagnosed brain tumors: five glioblastoma multiforme, two oligodendroglioma, one ependymoma, and one astrocytoma. PBMCs were incubated overnight with autologous tumor cells or autologous dendritic cells loaded with a U118 cell lysate, and responses were detected by IFN-γ ELISPOT and cytokine flow cytometry assays.

Results: PBMCs from all glioblastoma multiforme patients exhibited IFN-γ responses to autologous tumor but not to HLA-mismatched U118 cells. Glioblastoma multiforme–specific IFN-γ responses were primarily mediated by CD8+ T cells and represented ~2% of total CD8+ T cells. Additionally, all glioblastoma multiforme patients responded to autologous dendritic cells loaded with U118 lysate but not with low-grade astrocytoma cell lysates. PBMCs from four patients with other brain tumor types and one normal donor failed to respond to U118 lysate—loaded autologous dendritic cells. These data indicate that the IFN-γ responses to U118 lysate—loaded autologous dendritic cells are glioblastoma multiforme specific. Moreover, PBMCs stimulated 1 to 2 weeks with U118 lysate—loaded dendritic cells exhibited MHC class I–restricted cytotoxicity against autologous tumor cells.

Conclusions: Glioblastoma multiforme patients exhibit circulating tumor-specific CD8+ T cells that recognize shared tumor antigens from the glioblastoma multiforme cell line U118. These data show that glioblastoma multiformes are immunogenic and support the development of immunotherapy trials.

There is accumulating evidence to suggest that glioblastoma multiforme may be amenable to immune therapy approaches. Standard treatment for glioblastoma multiforme, which consists of surgical resection followed by radiation therapy, is associated with an average survival of less than 1 year (1). Chemotherapy has not been documented to improve survival (2). Glioblastoma multiformes exhibit varying degrees of infiltration with mononuclear cells, consisting primarily of T lymphocytes, suggesting the presence of a cell-mediated immune response (3). In several different animal models, antiglioma responses have been shown with in situ treatments with cytokines, such as IFN-γ, tumor necrosis factor-α (TNF-α), and interleukin (IL)-12 (4, 5), or with dendritic cells (6).

Several pilot studies with glioblastoma multiforme patients have described possible tumor responses to immunotherapies. Intratumoral injections of stimulated autologous lymphocytes have been associated with prolonged survival (7–9). Intradermal injections with autologous irradiated tumor cells plus granulocyte macrophage colony-stimulating factor, followed by collection of cells from draining lymph nodes and injection i.v., were associated with partial tumor responses in 4 of 12 patients (10). In another study, seven patients treated with intradermal injections of dendritic cells pulsed with peptides eluted from autologous tumor cells had prolonged survival compared with control patients (11). Additionally, in two of four patients in whom repeat tumor resections were done, CD8+ T-cell infiltration within tumors was documented.

However, virtually nothing is known about human glioblastoma multiforme–specific T-cell responses and their target proteins. Older studies using patient PBMCs were not definitive. PBMCs from glioblastoma multiforme patients were shown to inhibit the growth of autologous tumor cells at very high ratios, but responses did not seem to be specific because PBMCs from healthy donors also inhibited tumor cell growth (12, 13). In contrast to PBMCs, analyses of tumor-infiltrating lymphocytes were more revealing. Tumor-infiltrating lymphocytes from glioblastoma multiforme patient tumors were documented to represent predominantly CD8+ T cells (14). When expanded in microcultures, some tumor-infiltrating lymphocytes killed autologous tumor cells only, whereas other
tumor-infiltrating lymphocytes killed both autologous tumor cells and K562 cells, a natural killer (NK) cell target.

New, more sensitive methods are now available for the detection of human antigen-specific T-cell responses. In particular, the IFN-γ ELISPOT is a highly sensitive and quantitative method for the detection of tumor-specific T-cell responses (15). Moreover, this short-term assay avoids potential artifacts from prolonged incubation of PBMCs in vitro as well as inhibition by immunosuppressive factors such as transforming growth factor-β (TGF-β) and IL-10 secreted by tumor cells (16). However, this assay does not allow one to determine the phenotype or activation status of the producing cell. In contrast, the cytokine flow cytometry assay is less sensitive but allows discrimination of IFN-γ expression from activated CD4+ or CD8+ T cells (17). Additionally, methods have become available for the in vitro differentiation of dendritic cells from peripheral blood monocytes using cytokine cocktails (18). These monocyte-derived dendritic cells have been successfully used to present antigens from other tumor cells both in vitro as well as in clinical trials (19, 20).

The goal of this study was to develop a sensitive assay for the detection of tumor-specific T-cell responses from peripheral blood in patients with glioblastoma multiforme. Blood samples and autologous tumor cells were collected from patients with newly diagnosed brain tumors. Patient dendritic cells were prepared and used to present tumor antigens from the established glioblastoma multiforme cell line U118. PBMCs were used for T-cell responses to both autologous tumor cells and autologous dendritic cells pulsed with a cell lysate prepared from U118. The short-term IFN-γ ELISPOT and cytokine flow cytometry assays were used to detect and quantify T-cell responses.

Materials and Methods

Patient population. Buffy coat collections from one unit of blood were obtained through the Thomas Jefferson University Hospital Blood Center from nine patients with newly diagnosed brain tumors (GP1-GP9) after written informed consent was obtained. Separated plasma was also obtained from eight of the patients (except GP5). A buffy coat collection was obtained from one random blood donor as a normal control. Patient tumor tissues were obtained at the time of surgical resection. The Institutional Review Board approved the research protocol. Patient HLA class I typing was done by the Thomas Jefferson University Hospital Histocompatibility Laboratory.

Purification of peripheral blood mononuclear cells. PBMCs were separated from buffy coat collections using Ficoll-Hypaque density gradient centrifugation, as previously described (21). Cell aliquots were stored in liquid nitrogen in RPMI 1640 containing 10% DMSO and 45% autologous plasma (heat inactivated at 56°C for 30 minutes and passed through a 0.2 μm filter).

Preparation of autologous sera. Patient plasma was converted to serum by incubation with a 1:100 dilution of 1 mol/L CaCl₂ at 37°C for 18 to 24 hours to neutralize the anticoagulant and allow plasma to clot. Serum was transferred to a fresh tube and heat inactivated at 56°C for 30 minutes. Serum was centrifuged at 900 × g for 5 minutes, filtered through 0.45 and 0.2 μm filters to remove debris, and aliquots were frozen at –20°C.

Preparation of patient tumor cell lines. Tumor tissue from each patient was cut into small pieces with a sterile scalpel blade. Tumor fragments were suspended in 2 mL of a 0.25% Trypsin-EDTA solution (Gibco-BRL, Grand Island, NY) and pipetted up and down to break up large tissue fragments. After incubation for 5 minutes at 37°C, the suspension was again disaggregated and transferred to a 100 mm Primaria Petri dish (BD, Franklin Lakes, NJ) containing 10 mL of RPMI 1640/Opti-MEM (Gibco; 1:1) supplemented with 10% fetal bovine serum, 2 mmol/L glutamine, 100 units/mL of penicillin, and 100 μg/mL streptomycin. Cell lines were passaged three to five times before use.

Preparation of tumor cell lysates. Tumor cells (10⁴) were harvested, rinsed, resuspended in 2 mL HBBS, and lysed by five freeze/thaw cycles (dry ice/room temperature), followed by sonication for 2 minutes at 4°C to break up DNA. Debris was spun down at 400 × g for 25 minutes, and the supernatant was collected and stored in aliquots at –80°C.

Other cell lines. EBV-transformed B-cell lines (B-LCL) were prepared from PBMCs by incubation with supernatant from the EBV-infected marmoset cell line B95-81 [American Type Culture Collection (ATCC) CRL 1612] in the presence of cyclosporine A (1 μg/mL), as previously described (22). The human erythroleukemia cell line K562 was purchased from ATCC (Rockville, MD) and maintained in RPMI 1640 supplemented with 10% fetal bovine serum, 2 mmol/L glutamine, and penicillin/streptomycin. The human glioblastoma cell lines U118 (ATCC HTB-15) and LN-18 (ATCC CRL-2610) were purchased from the ATCC (Rockville, MD) and maintained in DMEM supplemented with 10% fetal bovine serum, glutamine, and penicillin/streptomycin. Both of these cell lines were previously HLA typed (23, 24).

Preparation of dendritic cells. PBMCs (30 × 10⁶ cells) were placed in a T25 flask in 5 mL of RPMI 1640 supplemented with 1% autologous serum (or human AB sera), 10 mmol/L HEPES, 2 mmol/L glutamine, and penicillin/streptomycin (R1), and incubated at 37°C for 90 minutes. The nonadherent fraction was aspirated, the flask was gently rinsed, and 5 mL of fresh R1 were added (day 0). On day 1, culture medium was aspirated and 5 mL of R1 containing granulocyte macrophage colony-stimulating factor (800 units/mL; Berlex, Wayne, NJ) and IL-4 (1,000 units/mL; BD PharmMingen, San Diego, CA) were added. Fresh cytokines were added again on day 3. Nonadherent cells (immature dendritic cells) were harvested on day 6. Dendritic cell aliquots (1 × 10⁶) were incubated with 100 μL tumor lysate in a final volume of 400 μL for 1.5 or 4 hours, infected with Ad 200 plaque-forming units/cell for 1.5 hours, or left untreated. Dendritic cells were rinsed, placed in six-well plates, and maturation was induction by the addition of 10 ng/mL IL-1β, 1,000 units/mL IL-6, 10 ng/mL TNF-α, and 1 μg/mL prostaglandin E2 (Sigma, St. Louis, MO) for 48 hours. Mature dendritic cells were confirmed to express the activation markers CD86, CD83, CD40, CD11c, and HLA DR by flow cytometry (data not shown).

Adenovirus. Subgroup C adenovirus type 5 virions were purified from virus-infected A549 cell lysates by an affinity chromatography method (Virapur, Carlsbad, CA) as per instructions of the manufacturer. The virion preparation was titrated by plaque assay as previously described (22).

Ex vivo ELISPOT assay. The IFN-γ ELISPOT assay was done, as previously described, using 96-well polystyrene microtiter plates (Millipore, Bedford, MA) with anti-IFN-γ monoclonal antibodies purchased from Mabtech (Stockholm, Sweden; ref. 25). PBMCs (250,000 cells) were incubated with each target (tumor cells or dendritic cells) at a 10:1 ratio in 100 μL RPMI supplemented with 10% pooled human AB sera (Atlanta Biologicals, Atlanta, GA), 10 mmol/L HEPES, 2 mmol/L glutamine, and penicillin/streptomycin (R10) in duplicate wells for 20 hours. Spot-forming cells were detected with a biotin-avidin alkaline phosphatase conjugate. Data were analyzed using a paired t test to compare the mean number of spots in the duplicate control and experimental microwells.

Cytokine flow cytometry assay. The cytokine flow cytometry assay was done using the BD Fast Immune CD8 and CD4 intracellular Cytokine Detection kits (BD Sciences, CA) as per the instructions of the manufacturer. PBMCs (0.5 × 10⁶-1 × 10⁶) were incubated with each target at a 1:1 ratio plus the mitogen phytohemagglutinin (10 μg/mL) and phorbol ester (10 ng/mL) as a positive control. Brefeldin A (5 μg/mL) was added to
each sample to prevent the secretion of newly synthesized IFN-γ, and cells were incubated at 37°C for another 4 hours. EDTA was added to remove adherent cells from the tube, and cells were suspended in BD FACS lysing solution and frozen at −80°C. Before staining, cells were thawed, washed, and incubated with BD FACS permeabilizing solution for 10 minutes at room temperature. After an additional wash, the mAbs anti–Hu-IFN-γ FITC, anti–CD69-phycocerythrin, anti–CD8 (or CD4) PerCP-Cy5.5, and anti–CD3-allophycocyanin were added in a single staining step and incubated for 30 minutes at room temperature in the dark. An aliquot of each sample was also fixed with 1% paraformaldehyde, and immediately analyzed by four-color immunofluorescence. Samples were collected on a FACS Calibur flow cytometer (BD, San Jose, CA) and analysis was done using CellQuest software.

**Cytotoxic T-cell assay.** Cytotoxicity was measured by a calcein release assay as previously described (26). In brief, targets were labeled with 5 μg/mL calcein (Molecular Probes, Eugene, OR) for 30 minutes at 37°C, extensively washed, and incubated with effectors in microrotter wells for 3 hours. Assays were done in triplicate using effector/target ratios ranging from 10:1 to 60:1. For blocking experiments, targets were preincubated 30 minutes with 40 μg/mL monoclonal antibody against monomorphic determinants on HLA class I (W6/32) and class II DR (clone L243) antigens (Leinco Technologies, St. Louis, MO). Cells were pelleted, 100 μL of each supernatant were transferred to new wells, and fluorescence was measured on a Victor^2^ 1420 multilabel counter (Wallace, Gaithersburg, MD). Spontaneous release was measured from target cells incubated in medium alone. Maximal release was measured from target cells incubated with 0.1% Triton in 50 mmol/L sodium borate, pH 9.0. Percent lysis is calculated as follows: [(sample fluorescence – SR fluorescence) / (MR fluorescence – SR fluorescence)] × 100, where SR is spontaneous release and MR is maximal release.

**Results**

**Glioblastoma multiforme peripheral blood mononuclear cells exhibit IFN-γ responses to autologous glioblastoma cells.** As an initial step, PBMCs from the glioblastoma multiforme patient GP1 (HLA A26, B39, B53) were directly screened for T-cell responses to autologous tumor cells. PBMCs were incubated with autologous tumor cells for 20 hours, and the number of IFN-γ–secreting cells was measured by ELISPOT assay, as described under Materials and Methods. This patient's tumor was a gliosarcoma with a glioblastoma multiforme component. As negative controls, PBMCs were incubated with the HLA-mismatched glioblastoma multiforme cell line U118 (HLA Aw24, A28, B12, Bw47) or media alone. As shown in Fig. 1A, GP1 PBMCs contained a large number of cells that produced IFN-γ in response to autologous tumor cells (>700 per 10^6 PBMC). In contrast, there was no response to HLA-mismatched U118 tumor cells, indicating that the IFN-γ response is MHC restricted. These data suggested the presence of a tumor-specific T-cell response in this patient.

**Patient tumor-specific T cells recognize autologous dendritic cells loaded with U118 lysate but not low-grade astrocytoma lysates.** Next, patient PBMCs were tested for the ability to recognize tumor antigens from the established glioblastoma multiforme cell line U118 presented by autologous dendritic cells. Immature dendritic cells were prepared from adherent PBMCs by incubation with granulocyte macrophage colony-stimulating factor and IL-4, as described under Materials and Methods. Immature dendritic cells were loaded with a U118 cell lysate for 1.5 or 4 hours, rinsed, and treated with IL-1β, IL-6, TNFs, and prostaglandin E2 for 48 hours to induce maturation. The U118 lysate–loaded mature dendritic cells were then incubated overnight with PBMCs and tested in the IFN-γ ELISPOT assay. As a positive control, PBMCs were incubated with dendritic cells infected with adenovirus (we have previously shown that nearly all individuals have memory T-cell responses to adenovirus; ref. 25). As negative controls, PBMCs were incubated with untreated dendritic cells or media alone. As shown in Fig. 1B, GP1 PBMCs responded to the adenovirus-infected dendritic cells positive control, confirming that the patient's dendritic cells are functional and that the patient exhibits memory antigen-specific T-cell responses. Moreover, PBMCs exhibited a very strong IFN-γ response to dendritic cells loaded with tumor lysate for 4 hours (but not 1.5 hours), consistent with the time course for uptake of a complex antigen source, such as tumor lysate, by immature dendritic cells. These data suggest that GP1 tumor-specific T cells recognize shared determinants on U118 antigens presented by autologous dendritic cells. Dendritic cells were loaded with tumor cell lysates for 4 hours in all subsequent experiments.

To further evaluate the specificity of the IFN-γ response, PBMCs from a second glioblastoma multiforme patient GP6 (HLA A2, A3, B8, B51) and a normal donor control were compared in ELISPOT assays similar to those done above.
The NK target K562 was included as an additional control because NK cells also produce IFN-γ. PBMCs from both donors responded to the adenovirus-infected dendritic cell positive control. Similar to GP1, GP6 PBMCs exhibited a strong IFN-γ response to autologous tumor cells (>700 per 10^6 PBMC) but did not respond to HLA-mismatched U118 cells, consistent with an MHC-restricted T-cell response. Additionally, GP6 responded to U118 lysate–loaded autologous dendritic cells. In contrast, there was a much lower response to the NK target K562 (136 per 10^6 PBMCs). In contrast to the glioblastoma multiforme patient, PBMCs from the normal donor had no detectable response to U118 lysate–loaded autologous dendritic cells. The fact that both glioblastoma multiforme patients exhibited MHC-restricted IFN-γ responses to autologous tumor and U118 lysate–loaded autologous dendritic cells, whereas the normal donor did not respond to U118 lysate–loaded autologous dendritic cells, confirmed that patients exhibit tumor-specific T-cell responses.

To rule out the possibility that these tumor-specific T-cell responses are targeted to normal astrocyte antigens shared by patient tumor cells and the U118 cell line, tumor lysates prepared from two different low-grade astrocytoma cell lines (a WHO grade 2 astrocytoma and a grade 1 pilocytic astrocytoma) were tested. Patient PBMCs failed to respond to autologous dendritic cells loaded with either low-grade astrocytoma lysate in the IFN-γ ELISPOT assay (data not shown). Additionally, patients did not respond to autologous dendritic cells loaded with an unrelated oligodendroglioma lysate (data not shown). Therefore, the IFN-γ response to U118 tumor lysate–loaded dendritic cells is specific for glioblastoma multiforme antigens shared by patient tumor cells and U118.

Five of five glioblastoma multiforme patients exhibit circulating tumor-specific IFN-γ responses. PBMCs from seven additional brain tumor patients were tested for responses to autologous tumor cells and U118 lysate–loaded autologous dendritic cells in the IFN-γ ELISPOT assay. Three of these patients (GP5, GP7, and GP9) had glioblastoma multiformes. Patient GP9’s tumor was a gliosarcoma with a glioblastoma multiforme component, similar to GP1. The remaining four patients had other tumor types—one ependymoma, one pilocytic astrocytoma, and two oligodendrogliomas. Data from all nine patients and the normal control are summarized in Table 1. All nine patients responded to adenovirus-infected dendritic cells as a positive control (average 337 per 10^6 PBMCs, range 45-680; data not shown). PBMCs from all five glioblastoma multiforme patients responded to both autologous tumor cells and U118 lysate–loaded autologous

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### Table 1. Comparison of IFN-γ antitumor responses in PBMCs from glioblastoma multiforme patients and patients with other brain tumor types

<table>
<thead>
<tr>
<th>UPN</th>
<th>Age, sex</th>
<th>Tumor type</th>
<th>IFN-γ SFCs per million PBMCs</th>
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</thead>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Autologous tumor</td>
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<tr>
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<td>GBM*</td>
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</tr>
<tr>
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<td>62, M</td>
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<td>GBM</td>
<td>&gt;700</td>
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<td>GBM</td>
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<tr>
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<td></td>
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</tbody>
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NOTE: PBMCs were incubated with each target at a 10:1 ratio for 20 hours, and IFN-γ–expressing cells were quantified by ELISPOT.

Abbreviations: UPN, unique patient number; SFCs, spot-forming cells; GBM, glioblastoma multiforme; Epend, ependymoma; Pilo, pilocytic astrocytoma; Oligo, oligodendroglioma; ND, not done.

*Gliosarcoma with a glioblastoma multiforme component.

>700, spots too numerous to count.
dendritic cells. In contrast, none of the four patients with other tumor types, including a related pilocytic astrocytoma, responded to U118 lysate–loaded dendritic cells. These data confirm that glioblastoma multiforme patients exhibit tumor-specific IFN-γ responses.

**Tumor-specific IFN-γ response is primarily mediated by CD8+ T cells.** To identify the cell type(s) responsible for the above tumor-specific responses, a cytokine flow cytometry assay was done. PBMCs from GP1 were incubated for 6 hours with U118 lysate–loaded or untreated autologous dendritic cells. Cocultures were done in the presence of Brefeldin A to prevent IFN-γ secretion. Cells were then stained with anti-CD3, anti-CD69, anti–IFN-γ, and either anti-CD4 or CD8 mAbs, as described under Materials and Methods. Dot-plots were defined by gating on either CD3+CD4+ cells (CD4+ T cells) or CD3+CD8+ cells (CD8+ T cells). As shown in Fig. 3, the tumor-specific IFN-γ response was primarily mediated by activated CD8+ T cells and represented 2.32% of total CD8+ T cells. In contrast, the IFN-γ response from CD4+ T cells was detected at a much lower level (0.10%). Of note, significant populations of both CD8+ and CD4+ T cells were activated (CD69+) but did not produce IFN-γ (top left quadrants).

Additionally, PBMCs from a different patient (GP6) were tested against autologous tumor cells using the cytokine flow cytometry assay as above. PBMCs were incubated with HLA-mismatched U118 cells or media alone as negative controls. Similar to the above experiment with GP1 PBMCs, using U118 lysate–loaded autologous dendritic cells, a CD8+ T-cell response to autologous tumor cells was detected from GP6 PBMCs, representing ~2% of the total CD8+ T-cell population (Fig. 4). In contrast, the CD4+ T-cell response to autologous tumor cells was <0.9% (data not shown). These data document that PBMCs from glioblastoma multiforme patients contain tumor-specific CD8+ T cells and that CD8+ T cells recognize tumor antigens shared with U118.

**Tumor-specific T-cell lines exhibit class I–restricted cytotoxicity.** To further evaluate the properties of the tumor-specific CD8+ T cells, tumor-specific T-cell lines were prepared and tested in a CTL assay. GP1 PBMCs were stimulated in vitro for 1 week with U118 lysate–loaded autologous dendritic cells to amplify tumor-specific T cells. Low-dose IL-2 (5 units/mL) was added at day 5. At day 7, the T-cell line was tested in a CTL assay against autologous tumor cells. As negative control targets, the NK target K562 and an autologous B-LCL line were tested. As shown in Fig. 5A, the T-cell line exhibited specific killing of autologous tumor cells only, consistent with MHC-restricted cytotoxicity.

To confirm this result, another tumor-specific T-cell line was prepared from a second donor for analysis. GP7 PBMCs (HLA A29, A68, B7, B53) were incubated with U118-loaded autologous dendritic cells for 1 week. IL-2 (5 units/mL) was added starting on day 5. Cells were restimulated with fresh U118-loaded dendritic cells on day 7 and tested by CTL assay on day 14. As shown in Fig. 5B, the T-cell line killed autologous tumor cells but failed to kill the HLA-mismatched glioblastoma multiforme cell line LN-18 (HLA A2, A9, B5, Bw35). Additionally, cytotoxicity was blocked by preincubation of targets with a mAb against HLA class I antigens but not class II antigens (Fig. 5C). Thus, the tumor-specific CD8+ T cells exhibit HLA class I–restricted cytotoxicity. Additionally, these studies illustrate that U118 lysate–loaded autologous dendritic cells can be used to stimulate tumor-specific cytotoxic CD8+ T cells in vitro.

**Discussion**

This is the first study to directly detect tumor-specific T-cell responses in peripheral blood from patients with newly diagnosed glioblastoma multiforme. Using a combination of highly sensitive, short-term IFN-γ ELISPOT assays and
quantitative flow cytometric analyses, MHC-restricted IFN-γ responses to autologous tumor cells were documented in PBMCs from all glioblastoma multiforme patients tested (4). Additionally, five of five glioblastoma multiforme patients exhibited specific IFN-γ responses to a tumor cell lysate from the established glioblastoma multiforme cell line U118 presented by autologous dendritic cells. The tumor-specific IFN-γ responses were mediated primarily by CD8+ T cells and represented ~2% of total CD8+ T cells.

Tumor-specific T cells from all five glioblastoma multiforme patients recognized U118 antigens presented by autologous dendritic cells. Dendritic cells are professional antigen-presenting cells. As such, they efficiently endocytose and present exogenous antigens, such as apoptotic cells, via both MHC class I and II molecules, a phenomenon known as cross-priming (27, 28). The T-cell response to U118 lysate–loaded dendritic cells was glioblastoma multiforme specific because patient PBMCs failed to respond to...
autologous dendritic cells loaded with lysates prepared from other tumor types (i.e., two different low-grade astrocytomas and an unrelated oligodendroglioma). There was no difference in the T-cell responses between the three patients with pure glioblastoma multiformes and the two patients with gliosarcomas with a glioblastoma multiforme component, suggesting that the patients with gliosarcomas exhibited T-cell responses to the glioblastoma multiforme component. Moreover, PBMCs from four patients with other brain tumor types did not recognize U118 lysate–loaded autologous dendritic cells. These data indicate that patient tumor cells and the glioblastoma multiforme cell line U118 share tumor antigens that are recognized by tumor-specific CD8+ T cells.

Tumor-specific IFN-γ responses were primarily mediated by CD8+ T cells, as shown by the cytokine flow cytometry assay. There was also a smaller but measurable population of tumor-specific CD4+ T cells producing IFN-γ (T-helper 1–like cells). Additionally, significant populations of CD8+ and CD4+ T cells expressed the T-cell activation marker CD69 but were negative for IFN-γ. These cells may be producing other cytokines in response to tumor antigens and warrant further investigation. In particular, tumor-specific T-helper 2–like CD4+ cells, producing cytokines such as IL-4 and IL-10, may be present and could serve to inhibit the T-helper 1 and CD8+ T-cell responses. This study does not address the role of antitumor immune responses mediated by B cells or the innate immune system. However, the glioblastoma multiforme cell line U118 was not recognized by lymphokine-activated killer cells in the CTL assay (data not shown).

Additionally, tumor-specific T-cell lines were successfully amplified from PBMCs from two glioblastoma multiforme patients by in vitro stimulation with U118 lysate–loaded autologous dendritic cells. Tumor-specific T cells exhibited MHC-restricted cytotoxicity against autologous tumor cells in a CTL assay. Cytotoxicity was confirmed to be mediated by HLA class I antigens by blocking experiments with mAbs against class I and class II antigens. Therefore, these studies document that U118 lysate–loaded dendritic cells can be used to present glioblastoma multiforme tumor antigens in vitro and amplify tumor-specific CD8+ T cells.

Of note, glioblastomas have properties in common with melanomas, another tumor cell type that is known to be immunogenic and to respond to immunotherapy. Glioblastoma multiformes arise from astrocytes, a cell type that originates from the neuroectoderm, as do melanomas. In fact, glioblastoma multiformes are known to express certain melanoma tumor antigens such as melanoma-associated antigens 1 and 3 and tyrosinase-related protein 2 (29). Therefore, T-cell responses may, in part, be targeted to melanoma tumor antigens, and further studies are ongoing.

If tumor-specific T cells are present in peripheral blood, then why are glioblastoma multiforme–specific T-cell responses ineffective in vivo? There are likely multiple factors involved. In particular, glioblastoma multiformes are known to secrete a number of immunosuppressive factors such as TGF-β, IL-10, and prostaglandin E2 (30–32). Glioblastoma multiformes also express Fas ligand (33), and it has been shown that other tumor cells that express Fas ligand may induce apoptosis of activated T cells (34). Additionally, investigators detected Fas-expressing apoptotic T cells within glioblastoma multiforme tissue (35). Therefore, T cells may be inactivated in situ (e.g., by immunosuppressive cytokines such as TGF-β or by induction of apoptosis by Fas ligand; ref. 36). Other possible factors include alterations in the blood-brain barrier induced by gliomas that may impede or prevent T-cell invasion. As an example, TNF-α is found in normal human cerebral endothelial cells and has been shown to induce the expression of adhesion molecules necessary for transmigration of immune cells through the blood-brain barrier (37). Gliomas seem to lack expression of TNF-α and, in fact, elaborate soluble TNF receptors which down-regulate infiltration of immune-competent cells (38). In addition, several studies have suggested that glioblastoma multiforme patients have T-cell dysfunction (39, 40). However, this is not supported by the current study because glioblastoma multiforme patients were found to exhibit memory adenovirus-specific T-cell responses, functional dendritic cells, and tumor-specific T-cell responses.

We have previously reported that s.c. injections of apoptotic autologous glioma cells induced antitumor responses in vivo, suggesting an immune-mediated response. In our pilot clinical trial, 8 of 12 patients with WHO grade 3 or 4 gliomas treated with apoptotic autologous tumors showed clinical improvement (41). Data from the present study document the presence of glioblastoma multiforme–specific CD8+ T-cell responses and support the hypothesis that these apoptotic tumor cells are taken up by dendritic cells in vivo and may boost an antitumor T-cell response. These studies also suggest the possibility that U118 lysate–loaded autologous dendritic cells may be used in lieu of autologous tumor cells in clinical trials.

In conclusion, these data indicate that glioblastoma multiformes are immunogenic and support a role for immunotherapy approaches. Immunotherapy could potentially generate new tumor-specific immune responses or enhance the efficacy of preexisting tumor-specific T-cell responses. The quantitative T-cell assays described in this report can be used to monitor tumor-specific T-cell responses in such clinical trials. In particular, generation of primary glioblastoma multiforme cell lines is labor intensive, and these lines generally expand poorly in tissue culture. Therefore, in the absence of known glioblastoma multiforme tumor antigens, these studies support the use of U118 lysate–loaded autologous dendritic cells for in vitro assays. Moreover, U118 could serve as a standardized antigen source for immunotherapy applications in lieu of autologous tumor cells. Additionally, given the HLA diversity within the general population, use of U118 lysate–loaded dendritic cells may be a more practical strategy for clinical trial development than stimulation with specific tumor antigens or peptides to which T-cell responses are restricted by specific HLA types.

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