Vaccination with Irradiated Tumor Cells Pulsed with an Adjuvant That Stimulates NKT Cells Is an Effective Treatment for Glioma

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Abstract

Purpose: The prognosis for patients with glioblastoma multiforme (GBM) remains extremely poor despite recent treatment advances. There is an urgent need to develop novel therapies for this disease.

Experimental Design: We used the implantable GL261 murine glioma model to investigate the therapeutic potential of a vaccine consisting of intravenous injection of irradiated whole tumor cells pulsed with the immuno-adjuvant α-galactosylceramide (α-GalCer).

Results: Vaccine treatment alone was highly effective in a prophylactic setting. In a more stringent therapeutic setting, administration of one dose of vaccine combined with depletion of regulatory T cells (Treg) resulted in 43% long-term survival and the disappearance of mass lesions detected by MRI. Mechanistically, the α-GalCer component was shown to act by stimulating "invariant" natural killer–like T cells (iNKT cells) in a CD1d-restricted manner, which in turn supported the development of a CD4+ T-cell–mediated adaptive immune response. Pulsing α-GalCer onto tumor cells avoided the profound iNKT cell anergy induced by free α-GalCer. To investigate the potential for clinical application of this vaccine, the number and function of iNKT cells was assessed in patients with GBM and shown to be similar to age-matched healthy volunteers. Furthermore, irradiated GBM tumor cells pulsed with α-GalCer were able to stimulate iNKT cells and augment a T-cell response in vitro.

Conclusions: Injection of irradiated tumor cells loaded with α-GalCer is a simple procedure that could provide effective immunotherapy for patients with high-grade glioma. Clin Cancer Res; 18(23): 1–14. ©2012 AACR.

Introduction

Glioblastoma multiforme (GBM) is a highly malignant primary brain tumor with an extremely poor prognosis. Despite recent advances in chemotherapy, median survival is only 15.4 months, and 5-year survival is less than 10% (1). There is therefore a pressing need to develop better treatments. Cellular immunotherapy is a potential therapeutic option for GBM, with the most commonly used approach being injection of autologous dendritic cells (DC) loaded ex vivo with unspecified antigens derived from autologous whole tumor preparations. However, although clinical trials have reported some success in eliciting T-cell–mediated antitumor immune responses, survival benefit in most studies has been modest (2).

The fundamental requirement of any active anticancer immunotherapy is the generation of potent systemic antitumor immune responses with long-lasting memory. In fact, unless antigen-presenting cells (APC) such as dendritic cells are properly licensed to initiate effector T-cell responses, vaccination can have a tolerizing effect (3). One way to promote optimal activity of dendritic cells is to include a potent immune adjuvant in the vaccine. α-Galactosylceramide (α-GalCer) is a glycolipid that stimulates invariant natural killer–like T cells (iNKT cells; ref. 4), a population of T cells with innate-like function found predominantly in spleen, liver, and bone marrow (5). In addition to cell surface markers typically observed on NK cells, iNKT cells express a semi-invariant αβ T-cell receptor that recognizes glycolipid antigens (including α-GalCer) bound to the MHC class I-like molecule CD1d. CD1d is expressed at high levels on APCs including dendritic cells, so inclusion of an iNKT cell ligand such as α-GalCer can encourage iNKT:DC interactions that enhance innate and
Translational Relevance

Alpha-galactosylceramide (α-GalCer) is a glycolipid that stimulates a population of natural killer–like T cells (NKT cells) to secrete cytokines and provide help to antigen-presenting cells (APC). It is therefore a promising adjuvant that could enhance the potency of anticancer vaccines. Here we show for the first time that a vaccine consisting of irradiated tumor cells pulsed with α-GalCer is an effective treatment of intracranial glioma in a mouse model. We also show that the NKT cell population required for this approach to succeed is present and functional in a group of patients with glioblastoma multiforme (GBM). In this respect, high-grade glioma seems to be an exception to other advanced solid cancers. This simple vaccine could be a useful treatment for a group of patients who currently face an extremely poor prognosis.

adaptive immune responses (6–8). Because CD1d is non-polymorphic, the “help” provided by iNKT cells to dendritic cells is not dependent on HLA status, making this attractive for broad clinical application. α-GalCer can also be combined with other adjuvants such as Toll-like receptor ligands to further strengthen vaccine-induced immune responses (9).

Anticancer vaccines that activate iNKT cells with α-GalCer are highly effective in animal studies, with long-lasting immunity induced in response to coadministration of tumor antigens and α-GalCer (10–13). This approach has yet to be tested directly in patients with cancer. One potential obstacle to clinical translation is the observation that iNKT cell numbers seem to be reduced in many patients with cancer (14, 15). In this study, we investigated the efficacy of a vaccine based on injection of whole tumor-derived antigens combined with α-GalCer in a murine glioma model. We also assessed whether the crucial effectors required for the success of α-GalCer–based vaccines are present and competent in a cohort of typical patients with GBM.

Materials and Methods

Mice

The inbred strain C57BL/6 was obtained from the Animal Resource Centre (Canning Vale, WA). Also used were CD1d<sup>−/−</sup> mice (16), MHC class II–deficient B6Aa<sup>α</sup>/Aa<sup>α</sup> mice (17), TAP1-deficient mice (18), and langerin-DTREGFP mice that express the human Diptheria toxin (DT) receptor under control of the langerin gene (19). In adoptive transfer experiments cells from OT-I × B6.SJL-PtprcaPepcb/BoyJ CD45.1<sup>+</sup> mice were transferred into C57BL/6 (CD45.2<sup>−</sup>) hosts as previously described (29). All mice were maintained by the Biomedical Research Unit, Malaghan Institute of Medical Research (Wellington, New Zealand). Experimental protocols were approved by Victoria University Animal Ethics Committee and done according to their guidelines. Mice were 6 to 10 weeks of age and matched for age and gender.

Cell lines and reagents

The murine glioma cell line GL261 was obtained from the DCTD Tumor Repository (National Cancer Institute, Frederick, MD, USA) and cultured in Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen) supplemented with 20% FBS (Sigma-Aldrich), 2 mmol/L GlutaMax, 100 U/mL penicillin, and 100 μg/mL streptomycin (all Invitrogen). A methylcholanthrene-induced fibrosarcoma cell line raised in a CD1d<sup>−/−</sup> mouse, and a CD1d-transfected C1R cell line, both used as controls in flow cytometry experiments, were gifts from Prof. Mark Smyth (Peter MacCallum Cancer Institute, University of Melbourne, Melbourne, Australia) and Prof. Vincenzo Cerundolo (Weatherall Institute of Molecular Medicine, University of Oxford, Oxford, United Kingdom), respectively. The iNKT cell ligand α-α-GalCer was manufactured by Industrial Research as previously described (20). Diphtheria toxin and doxorubicin were from Sigma-Aldrich. Recombinant human IFN-γ was from PeproTech.

Tumor implantation models

GL261 cells were harvested with TrypLE (Invitrogen) and washed. For subcutaneous implantation, 5 × 10<sup>5</sup> cells in 100 μL of DMEM were injected in the left flank. Mice were considered to harbor tumors when any 2 perpendicular diameters were both at least 2 mm. For intracranial implantation, 5 × 10<sup>5</sup> cells in 2 μL PBS were injected via a 32 gauge needle into the right striatum at a point 2.1 mm lateral to the bregma and at a depth of 3 mm using a stereotactic frame (Harvard Apparatus) under general anesthesia by intraperitoneal injection of 100 mg/kg ketamine and 10 mg/kg xylazine (both Phoenix Pharm). Lacrilube (Allergan) was applied to the cornea to prevent desiccation. Buprenorphine (Rencikk Benckiser Pharmaceuticals) and Carprofen (Norbrook Laboratories) were used for perioperative analgesia. Time to symptom appearance was defined as time to weight loss more than 10% or overt behavioral symptoms (reduced activity, hunching). The presence of brain tumors in symptomatic mice was confirmed by necropsy, including histologic examination in some experiments.

Generation and administration of whole tumor vaccine

To generate "Glioma/Gal" vaccines, GL261 cells were incubated in complete media supplemented with 200 ng/mL α-GalCer for 24 hours, γ-irradiated (150 Gy) on ice, washed 3 times in PBS, and resuspended in PBS for injection. The method for "Glioma/Gal/Dox" vaccines was identical except that 0.05 μmol/L doxorubicin was also added to the final 24 hours of culture. Unless otherwise stated, mice received 10<sup>5</sup> cells in 200 μL PBS, injected intravenously into a lateral tail vein. To mitigate toxicity when more than 10<sup>5</sup> cells were injected, mice were pretreated 10 minutes before vaccination with 2 U heparin (Pfizer) delivered intravenously. To deplete regulatory T cells (Treg), mice received...
125 μg of anti-CD25 antibody intraperitoneally (clone PC61, prepared in-house from hybridoma supernatant), a dose that was separately shown to deplete 40% to 50% of FoxP3+ CD4+ T cells in naive mice (data not shown).

Isolation of OT-1 cells and in vivo cross-priming assay

Spleens and lymph nodes from OT-1 \( \times \) B6.SJL-Ptpcr<sup>−</sup> Pepc<sup>−</sup>/BoyJ mice (CD45.1<sup>+</sup>) were transferred into C57BL/6 mice by intravenous injection and 1 day later recipient mice were injected intravenously with 200 μg endotoxin-free ovalbumin (OVA; Profos AG) and 200 ng α-GalCer. Seven days later mice were bled from the lateral tail vein and leukocytes stained directly ex vivo with antibodies for TCR V<sub>α</sub>2, CD8, and CD45.1. Flow cytometry was used to enumerate OT-1 cells as a proportion of total CD8+ lymphocytes.

In vivo depletion studies

CD8<sup>+</sup> cells were depleted with antibody clone 2.43, CD4<sup>+</sup> cells with clone GK1.5, and NK1.1<sup>+</sup> cells with clone PK136, all prepared in-house from hybridoma supernatants. Appropriate intraperitoneal dosing schedules were developed to achieve >95% depletion over the course of a given experiment. Depletion of langerin<sup>+</sup> cells in langerin- DTREGFP recipients was achieved with 350 ng of Diphtheria toxin given 48 and 24 hours before vaccination, and 1 and 2 days after vaccination. In some experiments, antigen presentation in spleen was prevented entirely by splenectomy conducted 7 days before vaccination. Splenectomies were conducted under general anesthesia as described by Reeves and colleagues (21).

Flow cytometry in animal experiments

All antibody staining was conducted for 10 minutes at 4°C in PBS supplemented with 1% fetal calf serum (FCS), 0.05% sodium azide, and 2 mmol/L EDTA. Nonspecific FcR-mediated binding was blocked by incubation for 10 minutes with anti-CD16/32 (clone 24G2, prepared in-house from hybridoma supernatant, and 200 ng α-GalCer. Seven days later mice were bled from the lateral tail vein and leukocytes stained directly ex vivo with antibodies for TCR V<sub>α</sub>2, CD8, and CD45.1. Flow cytometry was used to enumerate OT-1 cells as a proportion of total CD8<sup>+</sup> lymphocytes.

Analysis of cytokine release

Blood was collected from the lateral tail vein, allowed to clot at room temperature and serum collected after centrifugation. Levels of the cytokines interleukin (IL)-4, IL-12p70, and IFN-γ were assessed by cytokine bead arrays analysed on a Bio-Plex analyzer (Bio-Rad Laboratories). For analysis of cytokine release in spleen, liver, and lymph node postvaccination, tissue was removed from euthanized animals and mechanically dissociated. Enrichment for liver lymphocytes was conducted using a Lymphoprep gradient (Axis-Shield). Cells were washed and incubated at 37°C in IMDM with 5% FBS, 2 mmol/L GlutaMax, 100 U/mL penicillin, 100 μg/mL streptomycin, and 50 μmol/L 2-mercaptoethanol (all Invitrogen). Culture supernatants were collected after 48 hours for analysis.

MRI

MRI was conducted on anesthetized animals using a clinical 1.5-T MR scanner (Philips Medical Systems), equipped with a wrist solenoid coil. T1-weighted images were acquired with the following parameters: TE = 21.4 milliseconds, TR = 800 milliseconds, pixel size = 300 mm x 300 mm, thickness = 1 mm, 3 averages. Contrast was enhanced by intravenous administration of 100 μL/mouse of gadolinium-DTPA (Magnevist, Bayer Schering Pharma). T2-weighted spin-echo images were acquired with the parameters: TE = 54 milliseconds, TR = 2000 milliseconds.

Patient sample collection

Peripheral blood and tumor tissue was collected from 38 patients who met histologic criteria for a diagnosis of GBM. Exclusion criteria included any other active malignancy or any previous chemotherapy for a disease other than GBM. Venous blood was drawn into CPT tubes (BD Biosciences), the peripheral blood mononuclear cells (PBMC) collected according to manufacturer’s instructions, washed, and cryo-preserved in the vapor phase of liquid N2 in 70% RPMI 1640 media. PBMC were thawed, washed, and resuspended in PBS. Antibody staining was conducted at 4°C for 20 minutes in PBS. Fc receptors were blocked by incubation for 15 minutes with 2 mg/mL polyclonal human IgG (Intragam P, CSL Limited). Exclusion of dead cells was using LIVE/DEAD Fixable Blue (Molecular Probes), 4',6-diamidino-2-
phenylindole (DAPI; Molecular Probes), or propidium iodide (Sigma-Aldrich). Data were acquired on an LSRII flow cytometer or FACSCalibur apparatus (both BD Biosciences) and analyzed using FlowJo software (TreeStar Inc.). Compensation was conducted using the appropriate fluorophore-labeled antibodies bound to antimouse Ig-coated particles (BD Compbeads; BD Biosciences). For nonantibody stains, compensation was conducted using stained and unstained populations of positive control cells. Fluorescent monoclonal antibodies for the following molecules were used: CD1d (clone CD1d42; PE-conjugated), CD14 (MjP9; APC) and CD19 (SJ25C1; APC from BD Biosciences; CD3 (SK7; APC-H7), CD11c (Bu15; APC), CD16 (3G8; FITC), HLA-DR (L243; PerCP) from Biolegend. A lineage 1 cocktail with antibodies for CD3, CD14, CD16, CD19, CD20, and CD56 (BD Biosciences) was used to discriminate dendritic cells. Human iNKT cells were detected using CD1d monomers (NIH Tetramer Core Facility), that had been loaded with α-GalCer and tetramerized with streptavidin-PE (BD Biosciences).

**In vitro iNKT cell proliferation assay**

PBMCs were thawed, washed, and plated (5 × 10⁵ PBMCs per well in a 96-well plate) in IMDM supplemented with 5% human AB serum (Invitrogen), and either 100 ng/mL α-GalCer or vehicle. After 24 hours, 50 U/mL recombinant human IL-2 (Chiron Corporation) was added to each well. After 7 days, live cells were counted with Trypan Blue (Invitrogen) exclusion, and iNKT cells enumerated by flow cytometry.

**Analysis of impact of α-GalCer–loaded tumor cells on iNKT cell proliferation and allo-response**

Primary GBM cell cultures from 5 patients were either pulsed overnight with 200 ng/mL α-GalCer or left untreated, and then γ-irradiated on ice (150 Gy). After washing 3 times to remove any free α-GalCer, 10⁵ irradiated tumor cells were cocultured with 5 × 10⁵ carboxyfluorescein succinimidyl ester (CFSE)–labeled allogeneic PBMC in 96-well plates. After 5 days, CFSE dilution was assessed by flow cytometry.

**Assessment of MHC class II expression by GBM tumor cells**

Primary GBM cell lines were cultured for more than 48 hours with and without 100 U/mL recombinant human IFN-γ. Cells were assessed for HLA-DR expression by flow cytometry as described earlier.

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**Figure 1.** Glioma/Gal vaccine prevents glioma growth in a mouse model. A and B, mice were vaccinated intravenously with 10⁵ Glioma/Gal cells, unpulsed irradiated tumor cells, or with 200 ng free α-GalCer, and challenged with subcutaneous GL261 7 days later. C, mice were treated intravenously on day 0 with 10⁵ Glioma/Gal cells or 200 ng free α-GalCer, or received no treatment. On day 14 some groups were vaccinated intravenously with 200 μg OVA protein and 200 ng free α-GalCer. Plots (bottom) show individual mice and group means, with representative flow cytometry plots above. D, as for A except some mice were treated with Glioma/Gal/Dox cells. E, mice were vaccinated with 5 × 10⁵ Glioma/Gal/Dox cells, Glioma/Gal cells, or unpulsed irradiated tumor cells and challenged with intracranial GL261 tumor 7 days later. Four to 6 mice per group for all experiments. Data are representative of 2 or 3 independent experiments with similar results except C which was carried out once. “,” P < 0.05; “,” P < 0.01.
Figure 2. The α-GalCer–loaded whole tumor vaccine is dependent on the adjuvant function of activated iNKT cells. A, CD1d<sup>−/−</sup> mice or their CD1d<sup>+/+</sup> littermates were vaccinated with Glioma/Gal and challenged with GL261 subcutaneously 7 days later. Five mice per group, 1 of 3 independent experiments with similar results is shown. B, serum cytokine levels after intravenous administration of 200 ng free α-GalCer, 10<sup>5</sup> Glioma/Gal cells, or 10<sup>5</sup> irradiated tumour cells. Five mice per group, 1 experiment. C, serum cytokine levels after 5 × 10<sup>5</sup> Glioma/Gal cells administered intravenously to wild-type (WT) or CD1d<sup>−/−</sup> mice. Three to 5 mice per group, 1 experiment. D, splenocytes were harvested from WT mice 7 days after vaccination with either Glioma/Gal or irradiated tumor cells, and replated in 96-well plates in complete media without restimulation. Cytokine levels in the supernatant of splenocyte cultures were assessed 48 hours later. Three mice per group, 1 of 2 independent experiments with similar results is shown. E, CD1d surface expression by GL261 tumor cells in vitro. Positive and negative controls are WT CD11c<sup>+</sup> splenocytes and a fibrosarcoma cell line raised in a CD1d<sup>−/−</sup> mouse. *P < 0.05, **P < 0.01.

Statistical analyses

Bars and error bars depict means and standard error of the mean except in graphs of observational patient data (Fig. 6 and Supplementary Fig. S7), which depict medians and quartiles. Nonparametric tests were used to assess statistical significance. For comparisons of 1 variable, the Mann–Whitney test was used for unpaired data, the Wilcoxon–matched pairs test for paired data, and the Kruskal–Wallis 1-way analysis of variance (ANOVA) for experiments comparing more than 2 groups, with Dunn posttest to determine statistical significance between 2 individual groups. For comparisons of 2 variables, a 2-way ANOVA with Bonferroni posttest was used. A log-rank test was used to determine statistical significance between Kaplan–Meier survival curves. All statistical analyses were done with Prism 5.0 software (GraphPad Software, Inc.), with P values of < 0.05 considered significant (*P < 0.05; **P < 0.01; ***P < 0.001). In studies comparing patient groups, all subjects with complete data for that particular study were included. Age-matching was conducted by alternately removing the oldest or youngest subject from each group until the difference between the mean ages of the 2 groups was no more than 2.5 years.

Results

Prophylactic treatment with an α-GalCer–loaded whole tumor vaccine prevents glioma growth in a mouse model

Responses to vaccination were studied in a murine glioma model in which GL261 cells were implanted by intracranial or subcutaneous injection into immunocompetent syngeneic mice. To generate a vaccine, tumor cells were pulsed overnight with α-GalCer, irradiated, and then washed to remove excess α-GalCer (‘Glioma/Gal’ vaccine). Because INKT cells are found predominantly in spleen and liver, the vaccine was delivered intravenously to provide access to these populations. Unlike some rodent glioma cell lines (22), GL261 forms lethal tumors when implanted subcutaneously. The α-GalCer–pulsed cells were therefore irradiated to prevent tumor outgrowth and to potentially enhance their immunogenicity (23). When used in a prophylactic setting, the Glioma/Gal vaccine conferred significant protection against a subcutaneous challenge with
GL261 tumor cells (range, 60–100% of mice protected, mean 82%; Fig. 1A and D). Injection of free α-GalCer, or irradiated tumor cells alone, did not confer significant protection, suggesting that activation of iNKT cells with α-GalCer coordinates an improved response to the tumor antigens in the Glioma/Gal vaccine (Fig. 1A and B). A vaccine consisting of tumor lysate coadministered intravenously with free α-GalCer was equally effective at protecting against subcutaneous tumor challenge (data not shown).

However, free α-GalCer administered intravenously is known to cause iNKT cell anergy lasting several weeks (24), potentially restricting its application in prime-boost regimens. To see if the Glioma/Gal vaccine also induced anergy we vaccinated mice and 2 weeks later assessed
whether a second vaccination consisting of soluble chicken OVA and free α-GalCer could induce expansion of OVA-specific T cells, a process that depends on the activity of iNKT cells (6). Although prior exposure to 200 ng free α-GalCer completely abrogated the response to the second vaccine, pretreatment with the Glioma/Gal vaccine caused only partial attenuation (Fig. 1C). The chemotherapeutic agent doxorubicin has been reported to increase the immunogenicity of tumor cells (25) even at very low noncytotoxic doses (26). To exploit this phenomenon, we assessed treatment of α-GalCer–pulsed tumor cells in vitro with a low dose of doxorubicin before irradiation and vaccination. Doxorubicin treatment resulted in vaccines with a slightly higher proportion of necrotic cells (Supplementary Fig. S1). There was a trend for this Glioma/Gal/Dox vaccine to provide improved protection in comparison with the Glioma/Gal vaccine (Fig. 1D). Although this did not reach statistical significance, this was probably because the Glioma/Gal vaccine was already highly potent and we elected to use doxorubicin treatment of the vaccine in all intracranial tumor experiments. Accordingly, the Glioma/Gal/Dox vaccine was shown to confer excellent protection against subsequent intracranial tumour challenge (Fig. 1E).

The α-GalCer–loaded whole tumor vaccine is dependent on the adjuvant function of activated iNKT cells

iNKT cells were absolutely required for the Glioma/Gal vaccine to confer tumor immunity, as protection from
tumor challenge was not observed in CD1d<sup>−/−</sup> mice without iNKT cells (Fig. 2A). After intravenous treatment with free α-GalCer, the glycolipid is acquired by resident APCs and then presented via CD1d to activate iNKT cells (27, 28). Activation of iNKT cells provokes release of cytokines including IL-4, IL-13, and IFN-γ into the serum. The interaction with APCs is reciprocal, with the activated iNKT cells in turn stimulating the APCs to release IL-12p70. This interaction, which involves CD40/CD40L interactions, also promotes the priming of conventional T cells (6, 8). To examine whether similar interactions take place after intravenous administration of Glioma/Gal, serum was collected at different time points after vaccination to analyse cytokine secretion, and compared with animals that received free α-GalCer (Fig. 2B). As expected, in wild-type (WT) mice administered free α-GalCer, high levels of IL-4, IL-12p70, and IFN-γ were detected that peaked at 2, 12, and approximately 24 hours, respectively. In contrast, after infusion of Glioma/Gal the systemic release of these cytokines was much lower, and peaked later in the case of IL-4 and IL-12p70. Notably, cytokine secretion after Glioma/Gal infusion was absent in serum in CD1d<sup>−/−</sup> mice, confirming that iNKT activation triggered the cytokine release observed in WT animals (Fig. 2C). Although the levels of IL-12p70 were lower after Glioma/Gal compared with free α-GalCer (Fig. 2B), the ratio of peak IL-12p70 to IL-4 was higher (approximately 100:1 compared with 3:1), possibly indicating vaccine-induced APC activation with proportionately lower systemic iNKT cell activation. These observations suggest that α-GalCer is presented in a qualitatively different manner <em>in vivo</em> when incorporated into the vaccine. As GL261 tumor cells do not express CD1d (Fig. 2E), and are therefore incapable of stimulating iNKT cells directly, other CD1d<sup>+</sup> cell types must have acquired α-GalCer for presentation to iNKT cells. Cytokines were still being secreted in significant quantities from splenocytes isolated from mice 1 week after vaccination, which was not the case in mice that had received free α-GalCer (Fig. 2D).

The α-GalCer–loaded whole tumor vaccine elicits a CD4<sup>+</sup> T-cell–mediated immune response

We next defined the effector arms responsible for protection from tumor challenge. Depletion of NK1.1<sup>+</sup> cells after the priming phase did not abrogate protection (Supplementary Fig. S2A) suggesting that NK cells, which are typically activated immediately downstream of iNKT cell stimulation, were not important antitumor effectors. The Glioma/Gal vaccine was not capable of protecting against challenge with the unrelated Lewis Lung Carcinoma cell line LLC (Fig. 3A), indicating that vaccine-induced immunity was tumor specific. The Glioma/Gal vaccine also provided protection against late rechallenge with GL261 cells, indicating immunologic memory (Fig. 3B). Transfer of serum to naïve mice from vaccinated mice that had survived a tumor challenge did not confer protection, indicating that antibodies were not likely to be directly involved in protection (Supplementary Fig. S2B). Experiments carried out in animals depleted of specific T cell subsets showed that protection was lost in the absence of CD4<sup>+</sup> T cells, but retained in the absence of CD8<sup>+</sup> T cells (Fig. 3C). This was confirmed in knockout mice, with protection lost in MHC class II<sup>−/−</sup> hosts, which lack CD4<sup>+</sup> T cells, and retained in TAP1<sup>−/−</sup> hosts which lack CD8<sup>+</sup> T cells (Fig. 3D). It was independently shown that iNKT cells are present and able to respond to α-GalCer in these MHC class II<sup>−/−</sup> mice (data not shown). The dominant role of CD4<sup>+</sup> T cells was confirmed by the observation that IFN-γ secretion by splenocytes after vaccination was abrogated by addition of an MHC class II blocking antibody and by depletion of CD4<sup>+</sup> cells (Supplementary Fig. S3). Furthermore, supporting a lack of significant involvement of CD8<sup>+</sup> T cells in this vaccination strategy, protection was retained in the absence of CD8<sup>+</sup> T cells.
langerin$^+$ APC (Fig. 3E), a cell type recently shown to be critically involved in cross-presentation of blood-borne antigens to CD8$^+$ T cells (29, 30).

Given the requirement for CD4$^+$ T cells in conferring protection, it is notable that expression of MHC class II molecules by GL261 was negligible in the steady state but readily induced after exposure to IFN-$\gamma$ in vitro and spontaneously upregulated in vivo (Fig. 3F). It is possible that CD4$^+$ T cells directly target tumor cells via MHC class II, as has recently been suggested (31).

The $\alpha$-GalCer–loaded whole tumor vaccine primes adaptive responses in the lung-draining lymph nodes

Interestingly the Glioma/Gal vaccine conferred full protection against tumor challenge after splenectomy (Fig. 4A). Thus, despite the spleen being a significant source of the cytokines released after vaccination it was not required for effective immunity. Different anatomic sites were therefore examined for cytokine production to identify possible alternative locations of APCs mediating the protective immune response. High levels of cytokine production were detected in the mediastinal lymph nodes (MdLN) of vaccinated mice, but not in inguinal lymph nodes or liver (Fig. 4B). There was an increase in the overall size of MdLNs and a significant increase in the proportion of CD4$^+$ T cells expressing the activation marker CD44 in the MdLNs of immunized mice that was not seen in inguinal lymph nodes (Fig. 4C and D). In addition, we observed iNKT cell activation (Supplementary Fig. S4) and proliferation (Supplementary Fig. S5) and an increase in the proportion of MHC class II$^+$ CD11c$^+$ cells in the MdLNs of vaccinated mice (Fig. 4E). We speculate that the infused tumor cells are filtered by the lung capillary bed where they are taken up by lung-resident or circulating immune cells that traffic to lung-draining lymph nodes.

The $\alpha$-GalCer–loaded whole tumor vaccine is effective against established intracranial tumors but requires depletion of regulatory T cells

The efficacy of the Glioma/Gal vaccine was tested in a therapeutic setting where tumors were implanted intracranially, and mice vaccinated 6 days later. Although the vaccine provided effective protection against intracranial tumors in a prophylactic model (see Fig. 1E), in a therapeutic setting minimal or no protection was seen after vaccination in repeated experiments (Fig. 5A). However, in line with previous studies (32–34), we found that partial depletion of Tregs before vaccination resulted in effective treatment, with up to 60% long-term survival after a single vaccination (range, 29%–60%, mean 43%). The survival benefit could not be explained by regulatory T-cell depletion alone (Fig. 5B). MRI scans at 20 days postimplantation (14 days post-vaccination) showed enhancing mass lesions in most vaccinated mice that were radiologically similar to, but smaller than, those seen in unvaccinated mice (Supplementary Fig. S6A). Complete resolution of the lesion was documented in 1 vaccinated mouse that exhibited long-term survival (Fig. 5C). To confirm that radiologic abnormalities were tumors, some vaccinated mice were sacrificed on day 20 for histologic examination (Supplementary Fig. S6B). Overall, these data confirm that the vaccine is effective in prolonging survival and, in some cases eradicating established intracranial tumors.

Patients with GBM have functional iNKT cells that can respond to $\alpha$-GalCer–loaded GBM tumor cells in vitro

To succeed in the clinic, this vaccination strategy requires iNKT cells to be functional and present in sufficient numbers in patients with glioma. We therefore assessed iNKT number and function in the peripheral blood of 38 historically confirmed patients with GBM. Of these, 84% were newly presenting and 95% were taking the corticosteroid dexamethasone at the time of blood donation (mean daily dose 13.7 mg). No patients had received chemotherapy within 6 months. Because iNKT cell counts decline with age (35), the analysis in patients with GBM was compared with age-matched healthy controls. Flow cytometry with fluorescent $\alpha$-GalCer–loaded CD1d tetramers showed that the proportion of circulating T cells that were iNKT cells was slightly increased in the patient group (Fig. 6A). However, as previously described (36), the patients with GBM were lymphopenic relative to healthy controls, so that the absolute iNKT cell counts were not significantly different between the 2 groups (Fig. 6A, Supplementary Fig. S7). When we examined CD1d expression in some of the major APC populations in blood that could present myeloid dendritic cells (mDC) in all subjects with sufficient remaining PBMC, estimated by multiplying % mDC of total live PBMC (on flow cytometry). Top, solid gray is isotype, solid line is CD1d antibody. C1R-CD1d, CD1d-transfected monocytes. mDC were de

Figure 6. The cell populations required for $\alpha$-GalCer to be an effective adjuvant are preserved in patients with GBM. A, flow cytometry of peripheral blood from patients with histologically proven GBM and from an age-matched population of healthy volunteers, showing (left) iNKT cells as a percentage of total CD3$^+$ CD19$^+$ lymphocytes. Mean ages of the patient and control groups were 56.1 years (range, 44–74) and 58.6 years (range, 27–78), respectively. Middle, absolute iNKT cell number, estimated by multiplying % iNKT of total lymphocytes (on flow cytometry) and a contemporaneous automated peripheral blood lymphocyte count. Mean ages of the patient and control groups were 59.8 (range, 44–74) and 61.1 (range, 48–70), respectively. Right, absolute number of myeloid dendritic cells (mDC) in all subjects with sufficient remaining PBMC, estimated by multiplying % mDC of total live PBMC (on flow cytometry) and a contemporaneous automated peripheral blood count of lymphocytes + monocytes. mDC were defined as lineage marker low, CD11c high, HLA-DR high cells. Mean ages of the patient and control groups were 58 and 64 years, respectively. B, PBMC from patients with GBM and healthy volunteers were cultured in vitro with $\alpha$-GalCer for 7 days. Absolute iNKT cell number before and after coculture (left and center); fold change in absolute iNKT cell number (right). C, 5 GBM cell lines were pulsed or not pulsed overnight with $\alpha$-GalCer, then irradiated, washed, and cocultured for 5 days with CFSE-labeled PBMC from an allogeneic healthy volunteer. As a measure of proliferation, %CFSE cells of total cells was measured by flow cytometry. Left, representative flow cytometry plot of cell line 10/05, gated on live CD3$^+$ CD19$^+$ lymphocytes. Right, mean and SEM of triplicate wells for iNKT cells (top) and Tetramer $^+$ T cells (bottom). Bonferroni posttest P values of a 2-way ANOVA are shown on the lower graph. D, surface expression by GBM cell lines of CD1d (top) and HLA class II (HLA-DR) below as assessed by flow cytometry. Top, solid gray is isotype, solid line is CD1d antibody. C1R-CD1d, CD1d-transfected cell line for positive control. Bottom, solid gray is expression after culture in normal media; solid line, after 48 hours exposure to IFN-$\gamma$; broken line, isotype staining of IFN-$\gamma$-treated cells. *, P < 0.05; **, P < 0.01.
iNKT cells, patients had reduced numbers of circulating myeloid dendritic cells but monocyte numbers were preserved and CD1d expression by both cell types was equivalent to cells from healthy controls (Fig. 6A, Supplementary Fig. S7). Furthermore, iNKT cells could be expanded from PBMC of both patients and healthy controls in response to α-GalCer in vitro, albeit to a slightly reduced level in patients (Fig. 6B). Thus, the iNKT/DC axis seems to be sufficiently intact in patients with GBM for an α-GalCer–based vaccine to be effective.

To confirm this, α-GalCer was loaded onto primary tumor cell lines derived from 5 patients with GBM, which were then irradiated, washed, and cocultured for 5 days with CFSE-labeled PBMC from a healthy donor. Proliferation of donor iNKT cells was observed in all cases as loss of CFSE fluorescence on cells identified with α-GalCer–loaded CD1d tetramers (Fig. 6C). Proliferation of tetramer-negative cells was also observed, which occurs in this assay because of HLA-mismatch between tumor cells and donor PBMC. In 1 case this alloresponse was significantly enhanced when the tumor cells had been pulsed with α-GalCer, providing "proof-of-principle" that a vaccine based on α-GalCer–loaded tumor cells can enhance proliferation of non-CD1d restricted T cells as well as iNKT cells (Fig. 6C). The ability of α-GalCer–pulsed tumor cells to stimulate iNKT cells did not require the tumor to express CD1d because, as previously observed in the murine GL261 line, no surface staining of CD1d was seen on any of the primary GBM lines tested (Fig. 6D).

Finally, we confirmed that expression of MHC class II molecules after exposure to IFN-γ also occurred in the human primary GBM cell lines (Fig. 6D), which may therefore also be susceptible to direct targeting by CD4+ T cells.

**Discussion**

A simple vaccine composed of irradiated autologous tumor cells pulsed with α-GalCer, an adjuvant that stimulates iNKT cells, is an effective treatment against tumors in a murine glioma model. This vaccine design is also effective in murine models of melanoma and acute myeloid leukemia and is therefore not cell line–specific (unpublished data from our laboratory, with permission from T. Petersen, J. Gibbins, and C. Tan). The Glioma/Gal vaccine depends on the reciprocal activation of iNKT cells and APCs and elicits an adaptive immune response dominated by CD4+ T cells that is tumor-specific and long lasting. Although evidence of cellular activation was observed in the spleen, the MdLN was shown to be an important anatomical site of T-cell priming. We have further shown that the principal effectors necessary for the success of such a vaccine are present and functional in patients with high-grade glioma.

In some animal models, α-GalCer has been shown to have significant antitumor effects when injected intravenously as a sole agent (37). These effects were largely due to stimulation of the innate immune system, particularly NK cells (38), but enhancement of spontaneous adaptive responses was also observed in some tumor-bearing animals (39, 40). However, clinical trials in human patients with cancer, using either free α-GalCer or α-GalCer loaded onto dendritic cells, have achieved limited clinical benefit despite evidence of iNKT activation (7, 41, 42). All of these clinical trials used α-GalCer without coadministration of antigen. Thus, the adjuvant function of stimulating iNKT cells has yet to be tested directly in patients.

In animal models it has been reported that simple coadministration of α-GalCer with microbial products (43) or soluble antigens (6, 8) can significantly enhance antigen-specific T-cell–and antibody–mediated responses. There have also been reports that injection of α-GalCer with whole tumor cells can provoke effective antitumor responses, although different effectors were invoked depending on the model studied. As reported here for glioma, a previous report of an α-GalCer–pulsed lymphoma cell vaccine showed that efficacy was dependent on CD4+ T cells, and did not require CD8+ T cells (11). Similarly, CD4+ T cells could mediate full immunity in response to irradiated B16 melanoma cells administered intraperitoneally with free α-GalCer (10). In contrast, the antitumor activity of an α-GalCer–pulsed live B16 tumor cell vaccine was abrogated in both MHC class I−/− and MHC class II−/− animals (12). These contradictory findings suggest the type of response induced is a consequence of the tumor antigens presented and/or the timing and manner in which the infused tumor cells die. Shimizu and colleagues’ analysis of their live tumor cell vaccine showed that the injected α-GalCer–loaded tumor cells were killed by iNKT cells and NK cells, thereby providing a reservoir of antigens for uptake by resident APCs, with CD8α+ dendritic cells in spleen being important for cross-presentation (12, 44). In contrast, in the glioma study presented here, the tumor cells in the Glioma/Gal vaccine were CD1d-negative and, although alive at the time of injection, had been lethally irradiated. These different pathways to cell death are likely to result in differences in the timing of cell death and in the nature of the danger-associated molecular patterns and ligands for phagocytosis receptors expressed by the dying tumor cells. This in turn may determine the location, subset and maturation state of the APCs that take up the tumor cells and shape the immune response.

Because the Glioma/Gal vaccine was infused into the circulation, we anticipated a role for APCs in the spleen, and for CD8α+ dendritic cell in particular, as these APCs have been shown to have a selective capacity to take up and present antigen from circulating dying cells (12, 45). Surprisingly, in our model splenic dendritic cell were redundant. Indeed we found evidence that the MdLN was an important site of T-cell priming. Two major CD11c+ dendritic cell subsets have been described in the lung with recent studies suggesting the 2 populations have distinct functional roles (46). CD11b+CD103+ dendritic cells, which are langerin+, present antigen to CD8+ T cells. In contrast, CD11b−CD103− dendritic cells preferentially induce proliferation of CD4+ T cells (46, 47). Given that antitumor responses to Glioma/Gal were not abrogated when langerin+ cells were depleted, we speculate that infused tumor cells lodging in the pulmonary capillary bed...
might be preferentially taken up by CD11b<sup>hi</sup> CD103<sup>−</sup> dendritic cells that in turn generate the predominantly CD4<sup>+</sup> T-cell–mediated response observed. However, a role for other cell types of the monocyte/macrophage/dendritic cell lineage, either circulating in the blood or resident in the MdLNs, cannot be excluded. It has recently been reported that CD169<sup>+</sup> macrophages in the subcapsular sinus of lymph nodes are able to take up α-GalCer and activate iNKT cells (48), phagocyte dead tumor cells arriving via afferent lymphatics, and act as APCs to initiate antitumor immune responses (49).

Tregs progressively accumulate within GL261 tumors in vivo (50). Not surprisingly, in this study established intracranial tumors were resistant to therapeutic vaccination unless mice were also treated with an anti-CD25 depleting antibody. Although it has also been reported that activated iNKT cells can unexpectedly expand Tregs (51), it is unlikely that the Glioma/Gal vaccine induced Treg activity because Treg depletion was not required for successful prophylactic vaccination. In addition, we have previously shown that an α-GalCer–pulsed dendritic cell vaccine did not alter Treg number or function (13).

Tregs are just one of several immuno-suppressive and immunoevasion mechanisms hindering immunotherapy for high-grade glioma (52). In this study, 1 of 5 α-GalCer–pulsed GBM cell lines failed to stimulate iNKT cell expansion or elicit an allosresponse. Studies in progress suggest this might have been due to a tumor-derived soluble factor that causes a general inhibition of T-cell proliferation (data not shown). Attenuation of immuno-suppressive mechanisms will be a vital component of successful glioma immunotherapies in the future.

There is still a lack of identified glioma-associated antigens that are consistently expressed and/or capable of eliciting strong antitumor responses. For this reason, personalized vaccines incorporating autologous tumor extracts have been the most common immunotherapeutic strategy tested in patients with glioma. Other benefits of using whole tumor-based vaccines include applicability to all HLA types, the provision of MHC classes I and II epitopes, and the stimulation of a broad polyclonal response that reduces the risk of immune escape. Most studies of autologous tumor-based vaccines in patients with glioma have involved loading antigen onto autologous monocyte-derived dendritic cells. However, generating dendritic cells from patients with advanced disease can be problematic and there are many uncertainties surrounding the optimum phenotype of the infused dendritic cells (53). Thus, a vaccine that delivers whole tumor-derived antigens to endogenous dendritic cells may be a better approach than classical dendritic cell therapy, and intact autologous tumor cells constitute a ready-made vehicle for this purpose.

The use of irradiated whole tumor cells in anticancer vaccines raises a concern about possible induction of autoimmunity, particularly if the treatment also includes deliberate attenuation of peripheral tolerance mechanisms such as Treg. However, we saw no histologic evidence of immune cell infiltration in the normal brain parenchyma of treated mice distant from the tumor site (data not shown). Another concern is the risk of acute toxicity associated with the intravenous infusion of irradiated tumor cells. Toxicity encountered in this study at higher doses of the vaccine was effectively abrogated by pretreating mice with intravenous heparin, suggesting that tissue factor or some other pro-coagulant factor was responsible (54, 55).

Reduced numbers of iNKT cells and functional iNKT cell defects that could impact on the use of α-GalCer as an immune adjuvant have been reported in patients with advanced solid cancers (14, 15, 56). However, GBM seems to be an exception in this regard. A small study of iNKT cells in the blood of 9 patients with gliomas of various grades (57) reported no difference in iNKT cell function or number (expressed as percentage of total lymphocytes) compared with control subjects. Here a much larger and more homogeneous group of patients was examined, with iNKT cell number and function compared with age-matched healthy controls. All patients had a histologic diagnosis of GBM, and almost all were taking moderate to high doses of lymphotoxict corticosteroid. Importantly, we found no difference in the absolute iNKT cell count in peripheral blood in patients compared with controls, even though the patients were relatively lymphopenic. The relative sparing of iNKT cells in patients might reflect a greater resistance of this cell type to corticosteroid (58). We did find some deficiencies in the iNKT/DC axis in patients. A mild impairment of iNKT cell expansion might be explained by corticosteroid treatment (59). We also observed reduced numbers of circulating myeloid dendritic cells but circulating CD14<sup>+</sup> monocytes, known to be capable of differentiating into mature dendritic cells (60), were present in normal quantities and expressed normal levels of CD1d. Taken together, these data suggest there are no major defects in the iNKT/DC axis in patients with high-grade glioma and that α-GalCer–pulsed irradiated autologous glioma cells could be an effective vaccine in human patients.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Vaccination with Irradiated Tumor Cells Pulsed with an Adjuvant That Stimulates NKT Cells Is an Effective Treatment for Glioma

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