Human Cancer Biology

Clinical Cancer Research

Glioma-Associated Cancer-Initiating Cells Induce Immunosuppression

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Abstract

Purpose: Glioblastoma multiforme is a lethal cancer that responds poorly to therapy. Glioblastoma multiforme cancer-initiating cells have been shown to mediate resistance to both chemotherapy and radiation; however, it is unknown to what extent these cells contribute to the profound immunosuppression in glioblastoma multiforme patients and if strategies that alter their differentiation state can reduce this immunosuppression.

Experimental Design: We isolated a subpopulation of cells from glioblastoma multiforme that possessed the capacity for self-renewal, formed neurospheres *in vitro*, were capable of pluripotent differentiation, and could initiate tumors *in vivo*. The immune phenotype of these cells was characterized including the elaboration of immunosuppressive cytokines and chemokines by ELISA. Functional immunosuppressive properties were characterized based on the inhibition of T-cell proliferation and effector responses, triggering of T-cell apoptosis, and induction of FoxP3$^+$ regulatory T cells. On altering their differentiation state, the immunosuppressive phenotype and functional assays were reevaluated.

Results: We found that the cancer-initiating cells markedly inhibited T-cell proliferation and activation, induced regulatory T cells, and triggered T-cell apoptosis that was mediated by B7-H1 and soluble Galectin-3. These immunosuppressive properties were diminished on altering the differentiation of the cancer-initiating cells.

Conclusion: Cancer-initiating cells contribute to tumor evasion of the immunosurveillance and approaches that alter the differentiation state may have immunotherapeutic potential. *Clin Cancer Res; 16 (2); 461–73. ©2010 AACR.*

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Cancer-initiating cells are a heterogeneous population of undifferentiated cells with the capacity for self-renewal and a high proliferative potential. Treatments that are designed to eradicate tumors should also target the cancer-initiating cells (1). Glioblastoma multiforme, the most malignant among the adult human primary central nervous system tumors, contain these cancer-initiating cells that are multipotent and can recapitulate the characteristics of glioblastoma multiforme including high motility, diversity of progeny, tendency to migrate along white matter tracts, and expression of immature antigenic phenotypes such as epidermal growth factor receptor and nestin (2). Cancer-initiating cells may express CD133 (3), although cancer-initiating cells have been identified that do not express CD133 (4–6), form neurospheres that are nonadherent, have marker characteristics for all three astrocytic, neuronal, and oligodendroglial lineages (7), and are tumorigenic *in vivo*. These cells are also believed to confer the resistance to chemotherapy and radiation observed in glioblastoma multiforme patients (8, 9).

Malignant gliomas express tumor-associated and tumor-specific antigens that should make these tumors detectable to the immune system (10). However, there is a distinct lack of immunomediated tumor eradication in glioma patients, and most attempts at immunotherapy have met with little clinical success (11). Many factors work in concert to inhibit anti-glioma immunity, including immunosuppressive cytokines such as interleukin (IL)-10, transforming growth factor-β (TGF-β), and prostaglandin E_2, induction of regulatory T cells (Treg), and downmodulating costimulation molecules by antigen-presenting cells resulting in loss of T-cell effector function—all of which that have been shown to be operational in glioblastoma multiforme patients (reviewed in ref. 12). Although central nervous system tumors are recognized by the immune system, this is insufficient for their suppression or eradication. Primed CD8$^+$ cytotoxic T cells gain central nervous system access (13); however, the lack of tumor eradication

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Translational Relevance

Many malignancies, and especially glioblastoma multiforme, have notable chemotherapeutic and radiotherapeutic resistance, which is due, in part, to cancer-initiating cells capable of pluripotent differentiation and marked tumorigenesis. Glioblastoma multiforme patients are notable for profound immunosuppression and we hypothesized that glioblastoma multiforme–associated cancer-initiating cells contribute to the immunosuppression evident in these patients. This article shows that glioblastoma multiforme–associated cancer-initiating cells play a key role in mediating immunosuppression mechanistically by both cell-to-cell contact and secreted products, resulting in the inhibition T-cell activation and proliferation, induction of regulatory T cells, and initiation of T-cell apoptosis. These immunosuppressive properties are diminished on altering the differentiation state of the cancer-initiating cells. Thus, we propose the novel concept that strategies that induce the altered differentiation state of cancer-initiating cells could be used to reverse immunosuppression and as an immunotherapeutic approach.

indicates that the T cells are functionally impaired within the local tumor microenvironment. It is unknown whether the cancer-initiating cells within the glioblastoma multiforme participate in this tumor-mediated immunosuppression.

Only the immunosuppressive properties of human mesenchymal stem cells isolated from normal human donors have been characterized to date and these cells have been used in clinical trials for treatment of graft-versus-host disease (14). The mesenchymal stem cells typically have a spindle-shaped morphology and grow as single cells, express CD105 and CD90 but not CD133 (15), differentiate into mesenchymal cells, and are not tumorigenic *in vivo* but can enhance tumorigenesis of malignant cells (16). The mesenchymal stem cells express MHC-I but not the costimulatory molecules (17). Additionally, the mesenchymal stem cells induce dendritic cells to secrete IL-10, increase the number of Tregs, and decrease the secretion of IFN-γ from immune cells. This immunomodulation is mechanistically attributed to elevated prostaglandin E_2 levels and inhibitors of prostaglandin E_2 mitigated the immunosuppressive effects (18).

To understand the mechanism involved in glioma-mediated immunosuppression, we isolated cancer-initiating cells to ascertain if they possess similar immunosuppressive properties that influence the glioblastoma multiforme microenvironment. We hypothesized that the glioblastoma multiforme–associated cancer-initiating cells, by either direct cell-to-cell contact perhaps by expressing the costimulatory inhibitory molecule B7-H1 (19, 20) and/or by secreted immunosuppressive cytokines or factors (21, 22), would induce T-cell apoptosis (23–25) and/or the induction of Tregs (26, 27) that would inhibit T-cell proliferation. We then ascertained what would occur to the immunosuppressive properties of cancer-initiating cells if we altered their differentiation state as a potential approach to overcome cancer-initiating cell-mediated immunosuppression.

Materials and Methods

Human glioma cell lines. Human normal astrocytes and glioma cell lines U-251 and U-87 were purchased from the American Type Culture Collection and cultured in RPMI 1640 (astrocytes), MEM (U-251), or MEM plus 0.1 mmol/L nonessential amino acids (U-87). To all media, 10% fetal bovine serum and 1% penicillin-streptomycin were added.

Ethical treatment of research subjects and patient consent. Each patient provided written informed consent for tumor tissues and this study was conducted under protocol LAB03-0687, which was approved by the institutional review board of The University of Texas M. D. Anderson Cancer Center.

Human tumors. Tumor tissues from newly diagnosed glioblastoma multiforme patients (n = 9) were obtained from surgery specimens and graded pathologically according to the WHO classification system by a neuropathologist.

Human glioma-associated cancer-initiating cell derivation. Glioblastoma multiforme specimens were processed within 4 h after resection. They were washed with DMEM/F-12 and disassociated as described previously (9). Briefly, the tissues were enzymatically digested with Papain dissociation system (Worthington Biomedical). After a single-cell suspension was prepared, erythrocytes were lysed using 1× RBC lysis buffer (eBioscience). Trypan blue staining confirmed >80% cell viability. Dissociated tumor cells were cultured in DMEM/F-12 containing 20 ng/mL epidermal growth factor, basic fibroblast growth factor (Sigma), and B27 (1:50; Invitrogen) as a neural stem cell–permissive medium (neurosphere medium) at a density of 3 × 10^6 per 60-mm dish to form spheres. In parallel, single-cell suspensions from the glioblastoma multiforme specimens were cultured in U-87 medium (MEM) with or without differentiation factors (10 ng/mL retinoic acid and 20 ng/mL platelet-derived growth factor-AA, both from Sigma-Aldrich). After primary sphere formation was noted, sphere cells were dissociated for characterization of their properties as glioblastoma multiforme cancer-initiating cells such as immune phenotyping, cell self-renewal, differentiation, and tumorigenesis.

Neurosphere formation. Dissociated primary sphere cells were plated at a density of 500 per well in 24-well plates in 0.8 mL volumes of neurosphere medium. After 2 to 6 days, the neurospheres were formed for all glioblastoma multiforme specimens and the percentage of wells containing spheres ranged from 21% to 90%. The formation of neurospheres was maintained for multiple passages in neurosphere medium.

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Antibodies and reagents. Tissue culture–grade monoclonal antibodies to CD3 (OKT3) and CD28 (28.6) were obtained from eBioscience. Anti-human IL-6 (1936) and anti-human TGF-β1 (27235) antibodies were obtained from R&D Systems. Blocking antibody to B7-H1 and related isotype control IgG1 were purchased from eBioscience. The cell surface was stained with PE, FITC, or allo-phycocyanin–conjugated antibodies against the following proteins: CD3, CD4, CD8, MHC-I, MHC-II, CD40, CD80, CD86, and B7-H1 (BD Pharmingen) and CD133 (Miltenyi Biotech). To detect intracellular cytokines, PE-conjugated antibodies against IL-2 and IFN-γ (R&D Systems) were used. Appropriate isotype controls were used for each antibody. Recombinant human Galectin-3 was obtained from R&D Systems.

ELISAs. Supernatants from the glioblastoma multiforme tissue, the human glioma cell lines U-87 and U-251, and the glioma-associated cancer-initiating cells were measured for cytokine concentrations using ELISA kits as described (R&D Systems). These supernatants were collected from 3×10^6 cells after 5 days in culture and stored at −20°C. The supernatants were added in duplicate to appropriate precoated plates. After the plates were washed, horseradish peroxidase–conjugated detection antibody was added. The substrate used for color development was tetramethylbenzidine. The absorbance was measured at 450 nm with a microplate reader (Spectra Max 190; Molecular Devices), and chemokine concentrations were quantitated with SoftMax Pro software (Molecular Devices). The detection limits were 5 pg/mL for CCL-2, 16 pg/mL for TGF-β1, 5 pg/mL for IL-10, 1 pg/mL for IL-6, 10 pg/mL for prostaglandin E_2, 5 pg/mL for vascular endothelial growth factor, 10 pg/mL for Galectin-3, and 10 pg/mL for soluble Fas.

Human peripheral blood mononuclear cells. Peripheral blood mononuclear cells (PBMC) were prepared from healthy donor blood (Gulf Coast Blood Center) and glioblastoma multiforme patients' blood (the same patients whose cancer-initiating cells were isolated) by centrifugation on a Ficoll-Hypaque density gradient (Sigma-Aldrich)]. Aliquots of the isolated PBMCs were frozen and stored at −180°C until use. Before coculture experiments, frozen PBMCs were thawed at 37°C for 5 min and then washed with warm 10% fetal bovine serum in RPMI 1640. $CD3^+$ T cells were purified from PBMCs by negative selection using a Pan T-Cell Isolation Kit II (Miltenyi Biotech) according to the manufacturer's instructions.

Flow cytometry. FITC-conjugated anti-CD4 (RPA-T4) and antigen-presenting cells–conjugated anti-CD8 (RPA-T8) antibodies were used for cell surface staining. Subanalysis of the T-cell populations was based on the gated surface expression of CD4 and CD8. To detect FoxP3 protein expression, the surface-stained cells were further subjected to intracellular staining with PE-conjugated monoclonal antibodies to human FoxP3 (clone PCH101; eBiosciences) using staining buffers and conditions specified by the manufacturer. For intracellular cytokine staining, cells were stimulated for 6 h in the presence of 50 ng/mL phorbol myristate acetate, 500 ng/mL ionomycin (Sigma-Aldrich), and 2 μmol/L monensin (GolgiStop; BD Sciences). Then, the cells were incubated with FITC-conjugated anti-CD4 and antigen-presenting cells–conjugated anti-CD8 (RPA-T8) antibodies for surface staining followed by intracellular staining using PE-conjugated anti-mouse IFN-γ (4S.B3) or PE-conjugated anti-mouse IL-2 (MQ1-17H12) antibodies and FIX/PERM buffers (BD Pharmingen) according to the manufacturer's instructions. Flow cytometry acquisition was done with a FACSCalibur (Becton Dickinson) and data analysis was with FlowJo software (TreeStar).

Cell proliferation assay and Treg induction assay. The glioma-associated cancer-initiating cells and the differentiated glioma cells in the U-87 medium were plated into 48-well plates (3×10^4/mL) containing 3×10^5 PBMCs/mL in the presence of 1 μg/mL prebound anti-CD3/anti-CD28 antibodies or 2.5 μg/mL phytohemagglutinin (Sigma-Aldrich) with and without the B7-H1 (10 μg/mL) blocking antibody. Alternatively, conditioned media from the glioma-associated cancer-initiating cells were also added to the stimulated PBMCs. After 72 h, 100 μL of cells from each well were transferred to new 96-well plates with 10 μL Cell Counting Kit-8 (Dojindo Laboratories). After incubation for 4 h at 37°C, absorbance was measured at 450 nm with a microplate reader (Spectra Max 190). To detect $FoxP3^+$ Tregs, CD4 surface staining and then FoxP3 intracellular staining were done on immune cells cultured for 96 h.

$FoxP3^+$ Treg functional assay. Healthy donor PBMCs were labeled with 2 μmol/L carboxyfluorescein diacetate succinimidyl ester (CFSE) for 5 min at room temperature in PBS with 0.1% bovine serum albumin, and the reaction was quenched with RPMI 1640 with 10% fetal bovine serum for 10 min at 37°C. CFSE-labeled PBMCs (1×10^6/mL) and autologous T cells (1×10^6/mL), which were cultured with conditioned medium from glioma-associated cancer-initiating cells for 4 days, were plated into 96-well plates in the presence of allogeneic irradiated PBMCs (2×10^6/mL) in RPMI 1640 with 10% fetal bovine serum in a total volume of 0.2 mL. After 72 h, the cells were harvested, and analysis of cell division was done by flow cytometry.

Apoptosis assay. The T-cell apoptosis assay was done with the Annexin V/7-amino-actinomycin D staining kit (BD Pharmingen). Healthy donors PBMCs were cultured for 5 days with medium, glioma-associated cancer-initiating cells supernatants, or human Galactin-3 at 1 and 10 ng/mL and then harvested by centrifugation. Additionally, autologous glioblastoma multiforme patient PBMCs were cocultured with their respective glioma-associated cancer-initiating cells with and without anti–B7-H1 blocking antibody (10 μg/mL) at the beginning of the culture conditions in the cell contact–dependent apoptotic assay. The cells were stained with antigen-presenting cells–conjugated anti-CD3 antibodies and then washed twice with cold PBS and resuspended in 1× binding buffer (BD Pharmingen) at a concentration of 1×10^6/mL. Next, PE-conjugated

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Annexin V and 7-amino-actinomycin D were added, the cells were incubated for 20 min at 25°C in the dark, and CD3+ T-cell apoptosis was analyzed by flow cytometry within 1 h.

Cloning of single-cell cancer-initiating cells. After confirming the capacity for self-renewal, differentiation, and tumor formation at a low cell number, accutase (Sigma)–dissociated cancer-initiating cells were sorted using the CD133 cell isolation kit (Miltenyi Biotech) and >90% purity was obtained as detected by fluorescence-activated cell sorting (FACS). CD133+-sorted cells were seeded into 96-well plates at a theoretical density of 1 cell per well. After overnight culture, microscopic observation was used to identify wells that contained a single cell. These wells were monitored and the medium changed every 5 to 7 days for 45 days before immune functional analysis. *In vivo* tumorigenic potential were confirmed by formation of lethal tumor after intracranial implantation into nude mice.

Alteration of differentiation state of cancer-initiating cells. Accutase-dissociated sphere cells were cultured in differentiation medium consisting of 10% fetal bovine serum, 10 ng/mL retinoic acid, and 20 ng/mL platelet-derived growth factor-AA. Confluent monolayer cells were detached every 5 to 7 days by trypsinization, and retinoic acid and platelet-derived growth factor-AA were replenished during the culture. Similarly, the U-87 differentiated medium (MEM supplemented with 10 ng/mL retinoic acid and 20 ng/mL platelet-derived growth factor-AA) is used to differentiate total glioblastoma multiforme cells.

Immunohistochemistry. Differentiated cancer-initiating cells were cultured on eight-chamber slides (Nunc) at 5,000 per well. After 3 days, cells were fixed with 4% paraformaldehyde, permeabilized with 3% Triton X-100 in PBS, and then blocked with 5% horse serum. Primary antibodies were rabbit anti–glial fibrillary acidic protein (1:40; Dako), mouse anti-galactosylceramidase (1:100; Chemicon), and mouse anti–microtubule-associated protein 2 (1:50; Chemicon). After incubation for 90 min, the slides were washed with 5% horse serum. Secondary antibodies, goat anti-rabbit Alexa 546 (1:300; Invitrogen) and donkey anti-mouse Alexa 488 (1:300; Invitrogen) were added for 30 min. Slides were mounted using Vectashield Hard Set mounting medium with 4′,6-diamidino-2-phenylindole (Vector Laboratories).

Intracranial xenografting of cancer-initiating cells. Single-cell suspensions of glioma-associated cancer-initiating cells in serum-free medium at 1×10^3 cells/5 μL were injected into the right frontal lobes of 5- to 8-week-old nude mice (M. D. Anderson Cancer Center) using a stereotactic frame system (Kopf Instruments) as described previously (28). Animals were anesthetized with xylazine/ketamine during the procedure. Mice were maintained in the M. D. Anderson Isolation Facility in accordance with Laboratory Animal Resources Commission standards and conducted according to an approved protocol (08-06-11831).

Statistical analysis. All values were calculated as means and 95% confidence intervals from at least three independent experiments. The Student's *t* test was used to test for differences in the means between two groups. P values < 0.05 were considered to be statistically significant. All statistical analyses were done using the Statistical Package for the Social Sciences version 12.0.0 (SPSS). Error bars represent SD.

Results

Characterization of glioma-associated cancer-initiating cells. From newly diagnosed glioblastoma multiforme patients (n = 9) at the time of surgery, we isolated glioma-associated cancer-initiating cells and the patients' autologous T cells. The glioma-associated cancer-initiating cells from the patients expressed CD133 (range, 3-76%; mean, 32%; data not shown), formed neurospheres (Fig. 1A) in serum-free medium containing epidermal growth factor and basic fibroblast growth factor after 5 to 10 days of culture, and were capable of differentiating into glial fibrillary acidic protein astrocyte-like cells, neuron-like cells that were immunoreactive for microtubule-associated protein 2, and galactosylceramidase-immunoreactive oligodendrocyte-like cells (Fig. 1B). Furthermore, when the glioma-associated cancer-initiating cells (n = 3; 1,000 cells per mouse; 6 mice per glioma-associated cancer-initiating cells line) were injected in the right frontal lobes of 5- to 8-week-old nude mice, the mice developed tumors that were highly infiltrative along white matter tracts—a characteristic of human glioblastoma multiforme (Fig. 1C). After confirmation of their capacity for self renewal and recapitulation of the original tumor, the isolated glioma-associated cancer-initiating cells were used for the characterization of their immune properties.

Immunologic phenotype of glioma-associated cancer-initiating cells. To characterize their immunologic phenotype, the glioma-associated cancer-initiating cells (n = 5) were assessed for their expression of MHC-I, MHC-II, CD40, CD80, CD86, and B7-H1 by flow cytometry. The glioma-associated cancer-initiating cells expressed high levels of MHC-I (mean, 99.3%; range, 98.5-99.8%) and low levels of CD86 (mean, 6.7%; range, 5.9-7.9%), but not CD40 (mean, 5.8%; range, 0.7-15.8%), MHC-II (mean, 2.4%; range, 1.6-3.2%) or CD80 (mean, 0.6%; range, 0.2-0.6%; a representative example is shown in Fig. 2A), indicating that glioma-associated cancer-initiating cells lack the capacity for antigen presentation necessary to stimulate T-cell activation or proliferation. Furthermore, the inhibitory costimulatory molecule B7-H1 (mean, 31.2%; range, 28.5-34.9%) was expressed, indicating that direct contact between T cells and glioma-associated cancer-initiating cells would be inhibitory on immune cells.

Glioma-associated cancer-initiating cells produce immunosuppressive cytokines. To determine if the glioma-associated cancer-initiating cells produce immunosuppressive cytokines, glioma-associated cancer-initiating cells (n = 4) were assayed for immunosuppressive cytokines by ELISA. The glioma-associated cancer-initiating cells did not produce any appreciable IL-6, IL-10, soluble Fas, or tumor necrosis factor–related apoptosis-inducing ligand but did produce

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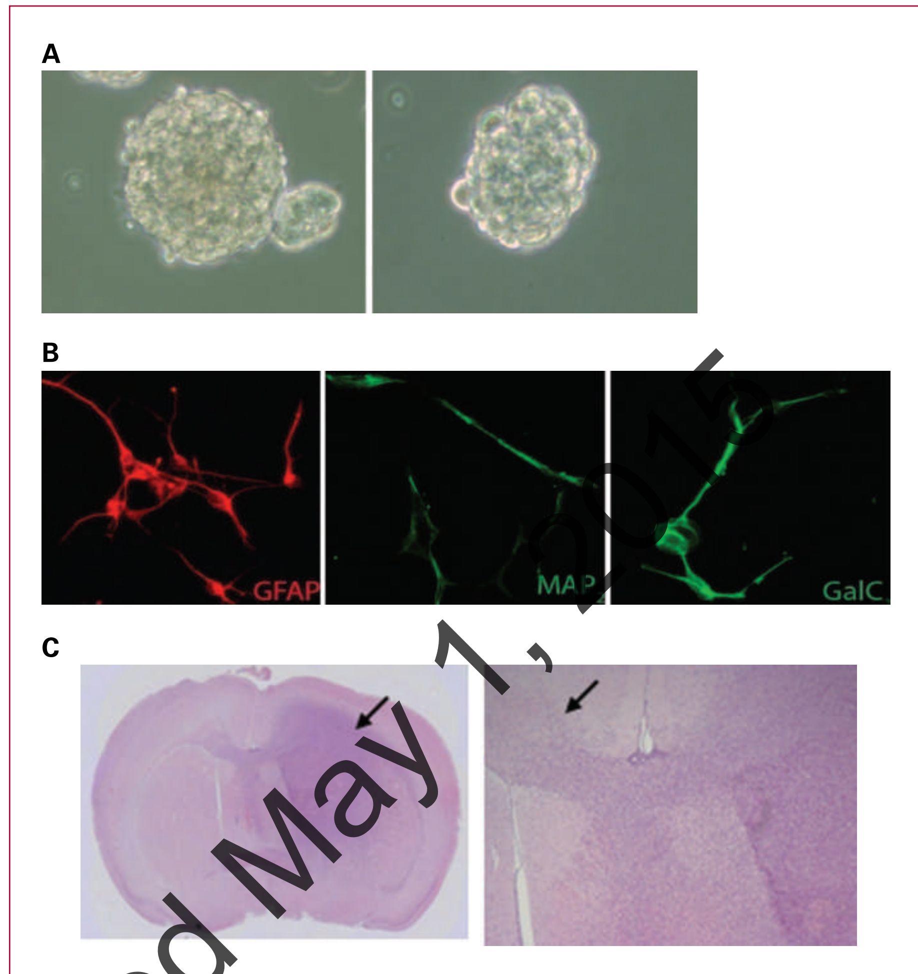

Fig. 1. Characterization of human glioma-associated cancer-initiating cells from glioblastoma multiforme specimens. *A,* a representative image of neurospheres from one glioma-associated cancer-initiating cells. *B,* after 7 days of culture in differentiating medium, the glioma-associated cancer-initiating cells differentiated into glial fibrillary acidic protein$^+$ astroglial lineage cells, microtubule-associated protein 2$^+$ neuronal lineage cells, and galactosylceramidase$^+$ oligodendroglial lineage cells (magnification, ×40), indicating that the glioma-associated cancer-initiating cells have multipotent differentiation potential. *C,* a representative image of a glioma-associated cancer-initiating cells xenografted into the frontal lobe of a nude mouse. The tumor that developed from the glioma-associated cancer-initiating cells caused enlargement of the brain and was diffusely infiltrative (*arrow*), including into white matter tracts such as the corpus callosum (*arrow*).

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TGF-β1 (24-73.8 pg/10^6 cells/24 h), the Treg chemokine attractant CCL-2 (8-710 pg/10^6 cells/24 h), vascular endothelial growth factor (14-61 pg/10^6 cells/24 h), and prostaglandin E_2 (34-60 pg/10^6 cells/24 h).

Glioma-associated cancer-initiating cells inhibit T-cell activation and proliferation. To determine if the glioma-associated cancer-initiating cells produce factors that would inhibit the activation and subsequent proliferation of immune cells, PBMCs from healthy donors were activated with anti-CD3/CD28 or phytohemagglutinin in the presence of conditioned medium obtained from 3-day cultures of glioma-associated cancer-initiating cells and T-cell proliferation was assessed by flow cytometry. The medium from a representative glioma-associated cancer-initiating cell was capable of inhibiting T-cell proliferation (Fig. 2B). This inhibition was seen regardless of the mechanism of stimulation (anti-CD3/CD28 or phytohemagglutinin); however, no inhibition of T-cell proliferation was detected when the conditioned medium was obtained from normal human astrocytes or the U-87 cell line (Table 1). To further show that individual glioma-associated cancer-initiating cells were capable of clonogenic growth and immunosuppression, CD133$^+$ cells were sorted from neurospheres and diluted for single-colony formation. Over 80% of seeded single cells grew out and 10 clones from two different neurospheres were selected at random and expanded for further immunologic characterization. As shown in Table 1, conditioned medium of all clonogenic lines displayed potent immunosuppression on CD3$^+$ T cells of normal donors, which potently inhibited T-cell proliferation by 91 ± 12% (P = 0.0005).

Coculture experiments with matched autologous PBMCs and the glioma-associated cancer-initiating cells also showed inhibition of T-cell proliferation (Fig. 2C), and this inhibition was partially reversed by the addition of B7-H1 blocking antibody in autologous coculture assays, indicating that B7-H1–mediated cell-to-cell contact is a mechanism contributing to cancer-initiating cells–mediated inhibition of T-cell proliferation (Fig. 2D). To further characterize this inhibition of immune cell proliferation, the glioma-associated cancer-initiating cells supernatants were coincubated with healthy donors' PBMCs in the presence of anti-CD3/CD28 stimulation, and the percentages of CD4$^+$ and CD8$^+$ T cells producing IL-2 and IFN-γ effector cytokines were determined by intracellular staining via

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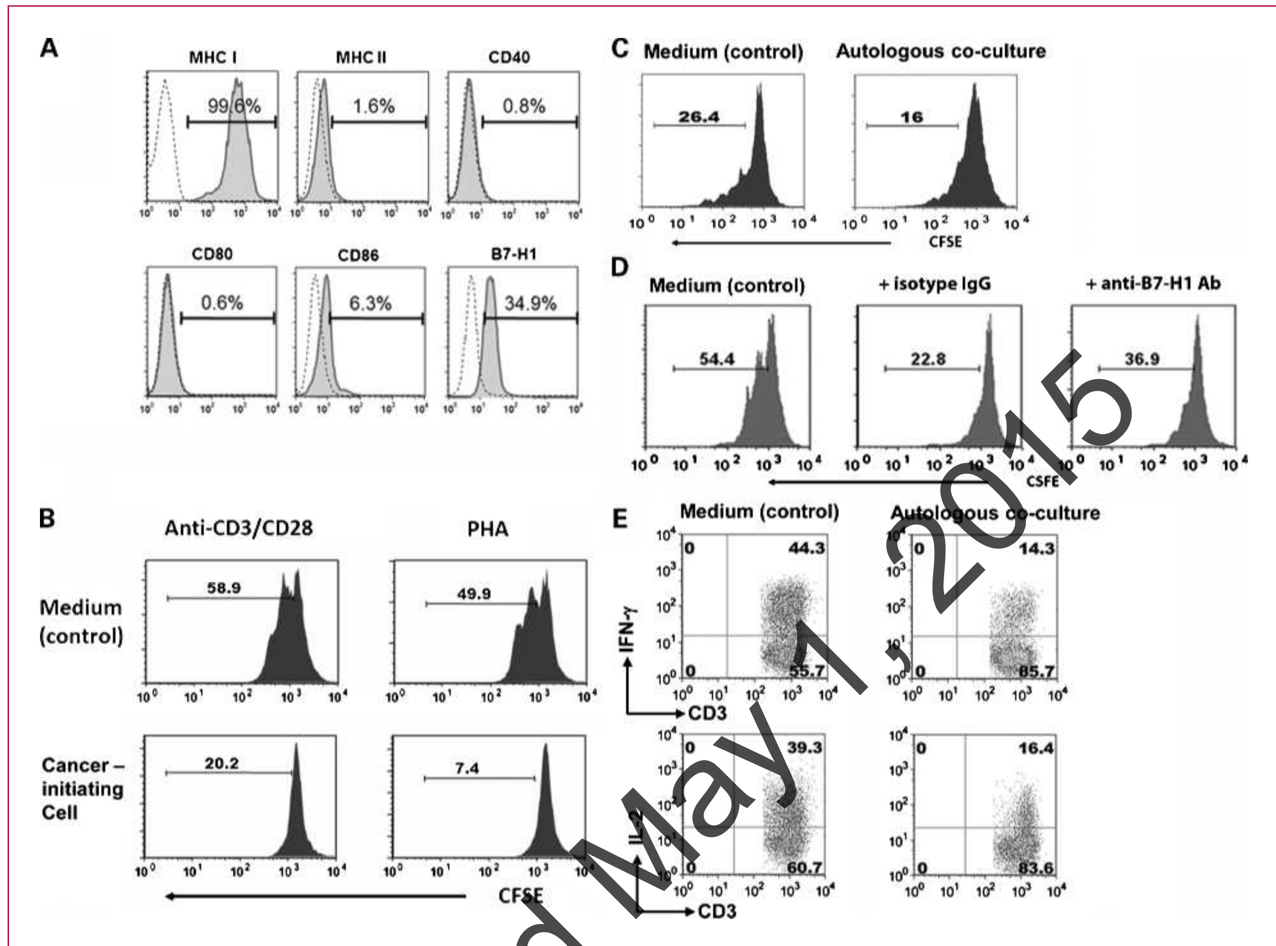

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Fig. 2. Glioma-associated cancer-initiating cells mediate immunosuppression on human T cells. *A,* immune surface phenotype of representative glioma-associated cancer-initiating cells. The glioma-associated cancer-initiating cells were surface stained with antibodies to MHC-I, MHC-II, CD40, CD80, CD86, and B7-H1. Representative FACS histogram plots for one glioma-associated cancer-initiating cell are shown for target staining (*solid line*) with associated isotype controls (*dotted line*). Percentages of the positive populations are shown. *B,* glioma-associated cancer-initiating cells supernatants inhibit T-cell proliferation regardless of activating stimulus. When healthy donor PBMCs were cultured in the presence of the glioma-associated cancer-initiating cells supernatants, T-cell proliferation was inhibited as shown by FACS analysis of T-cell CFSE labeling. Representative FACS histogram plots with the percentages of the indicated cells are shown. *C,* glioma-associated cancer-initiating cells suppress T-cell proliferation through cell-to-cell contact. When autologous PBMCs were cultured in the presence of glioma-associated cancer-initiating cells from the same glioblastoma multiforme patient, proliferation of T cells was inhibited as shown by FACS analysis with CFSE labeling. A representative FACS histogram plot is shown and similar results were obtained with glioma-associated cancer-initiating cells and matched PBMCs from two other patients. Percentages of the indicated populations of proliferated CSFE-labeled T cells are shown. *D,* B7-H1 expressed on glioma-associated cancer-initiating cells mediates suppression of T-cell proliferation through cell-to-cell contact. When autologous PBMCs were cultured with cancer-initiating cells of the same glioblastoma multiforme patient in the presence of B7-H1 neutralizing antibody, the inhibition of T-cell proliferation was partially reversed by B7-H1 blockade. The addition of isotype control IgG failed to suppress the inhibition of T-cell proliferation, similar to autologous coculture without IgG as shown in Fig. 2C. *E,* glioma-associated cancer-initiating cells inhibit T-cell function by downregulating effector cytokine production through cell-to-cell contact. After coculturing with autologous glioma-associated cancer-initiating cells for 3 days, glioblastoma multiforme patients' PBMCs were stimulated with anti-CD3/anti-CD28, surface-stained with anti-CD3, and then stained to detect intracellular IFN-γ and IL-2. Data were collected via FACScan. Compared with the medium (control), the glioma-associated cancer-initiating cells inhibited both IFN-γ and IL-2 production in gated $CD3^+$ T cells. One representative FACS plot is shown with percentage in the top right quadrant indicating percentage of positive cells. Similar results were obtained for glioma-associated cancer-initiating cells and matched PBMCs from two other patients.

flow cytometry. The number of IFN-γ and IL-2 producing CD4 and CD8 T cells were both reduced by 30% and 10%, respectively, by the cancer-initiating cell supernatants (n = 3). Similarly, the glioma-associated cancer-initiating cells inhibited IFN-γ and IL-2 generation by autologous $CD3^+$ T cells in cell-to-cell contact experiments (Fig. 2E). These data show that glioma-associated cancer-initiating cells suppress T-cell proliferative and proinflammatory responses.

Glioma-associated cancer-initiating cells induce Tregs. To determine if the decrease in T-cell proliferation and effector response was secondary to the TGF-β–producing glioma-associated cancer-initiating cells inducing Tregs, we ascertained the ability of the cancer-initiating cells to

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induce Tregs. Incubation with supernatants from the glioma-associated cancer-initiating cells markedly expanded the number of CD4$^+$FoxP3$^+$ Tregs in healthy donor PBMCs by 128 ± 51% ($P = 0.0007$; Table 1; representative example in Fig. 3A). These FoxP3$^+$ Tregs are functionally suppressive on autologous T-cell proliferation (Fig. 3B). Furthermore, the glioma-associated cancer-initiating cells induced FoxP3$^+$ Tregs in coculture experiments with PBMCs from each respective autologous glioblastoma multiforme patient (Fig. 3C). In this cell-to-cell contact context, addition of B7-H1 neutralizing antibody partially blocked the induction of FoxP3$^+$ Tregs (Fig. 2D), indicating that not only secreted factor(s) but also a cell-to-cell contact mechanism mediated by B7-H1 on the surface of cancer-initiating cells play a role in cancer-initiating cell-mediated immunosuppression. Conditioned medium from the U-87 glioma cell line did not induce Tregs, but the medium from astrocytes could but to a much lesser degree than the glioma-associated cancer-initiating cells (Table 1).

Table 1. Characterization of immunosuppressive properties of cloned cancer-initiating cells

Neurosphere parental line	Clone number	CD133$^+$ (%)	CFSE-labeled dividing T cells (%)	Change compared with medium (%)	Apoptotic T cells (%)	Change compared with medium (%)	Foxp3$^+$ Tregs (%)	Change compared with medium (%)
6-15	1	25.9	6.6	↓92	60.7	↑163	40	↑205
	2	88.9	3.8	↓95	48.9	↑112	27.8	↑112
	3	84.5	3.2	↓96	47.5	↑106	31.7	↑142
	4	47	7.2	↓91	55.8	↑142	35.9	↑174
	5	34.6	46.7	↓42	51.7	↑124	36.1	↑176
	6	89.6	3.1	↓96	53.4	↑131	24.4	↑86
	7	86.3	3.5	↓96	55.6	↑141	35.7	↑173
	8	84.7	5.7	↓93	61.1	↑165	44	↑236
	9	64	3.7	↓96	66.5	↑188	29	↑121
	10	70.2	4	↓95	67.3	↑191	31.7	↑142
11-28	1	97.5	4.1	↓95	47.8	↑107	24.2	↑85
	2	93.5	4.8	↓94	64.1	↑177	18.6	↑42
	3	76	5.6	↓93	54.5	↑136	28.7	↑119
	4	68.4	11.2	↓86	51.5	↑123	21.6	↑65
	5	93.6	5.6	↓93	55.9	↑142	21.6	↑65
	6	91.1	1.4	↓98	70	↑203	11.8	↓10
	7	81.5	5.2	↓94	54.5	↑136	29.8	↑127
	8	96.5	4.3	↓95	52.2	↑126	28.3	↑116
	9	96.2	6.1	↓92	50.6	↑119	29.6	↑126
	10	81.1	3.9	↓95	57.6	↑149	13.3	↑1.5
Control (cancer-initiating) medium	None	None	81	—	23.1	—	13.1	—
U-87	None	None	55.2	↓12	15.7	↓10	8.9	↓8
U-87 medium	None	None	63.3	—	17.4	—	9.7	—
U-87 cells propagated in cancer-initiating medium	None	None	82.9	↑2	21.8	↓6	14.5	↑11
Astrocyte	None	None	42.7	↓8	10.9	↓7	10.6	↑36
Astrocyte medium	None	None	46.4	—	11.7	—	7.8	—
U-87 medium*	None	None	34.1	—	29.3	—	11.5	—
Glioblastoma multiforme cell suspension in U-87 medium*	None	—	14.8	↓56	51.2	↑78	22.7	↑97
Glioblastoma multiforme cell suspension in U-87 differentiation medium*	None	—	28.7	↓16	33.8	↑15	10.9	↓5

*Contact-dependent coculture with autologous PBMCs.

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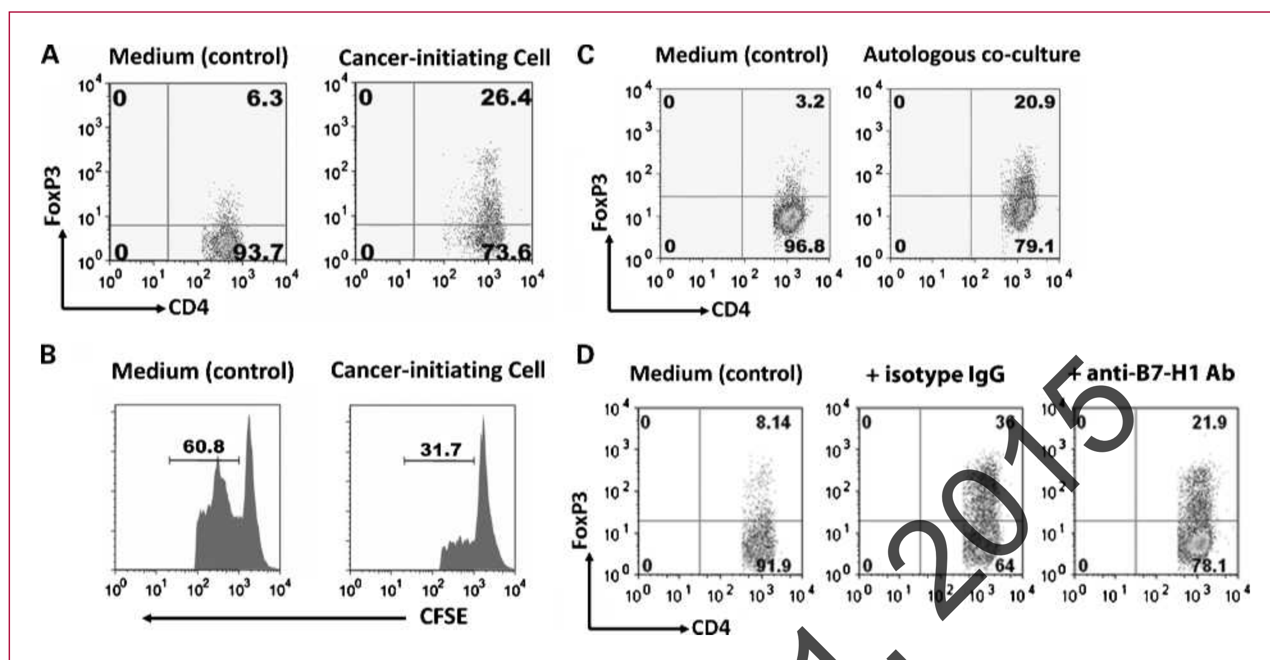

Fig. 3. Glioma-associated cancer-initiating cells induce functional Tregs. *A*, supernatants from the glioma-associated cancer-initiating cells induce an increase of the number of FoxP3$^+$ Tregs in the gated CD4$^+$ T cells as shown by the representative FACS analysis. *B*, FoxP3$^+$ Tregs induced by the supernatants of cancer-initiating cells suppress T-cell proliferation. T cells that were treated with cancer-initiating cell supernatants were harvested, cocultured for 3 days with autologous PBMCs (labeled with CFSE, responder cells) at a 1:1 ratio in the presence of soluble anti-CD3, and subsequently analyzed via FACScan. The number above the line in each histogram represents proliferating responder cells. *C*, glioma-associated cancer-initiating cells also induce FoxP3$^+$ Tregs through cell-to-cell contact. FACS analysis shows that the glioma-associated cancer-initiating cells increased the percentage of FoxP3$^+$ Tregs in the gated CD4$^+$ T cells. *D*, induction of FoxP3$^+$ Tregs mediated by cell-to-cell contact is partially reduced after the addition of B7-H1 neutralizing antibody. One representative FACS plot is shown with percentage in the top right quadrant indicating percentage of positive cells. Similar results were obtained for glioma-associated cancer-initiating cells and autologous PBMCs from two other patients.

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Glioma-associated cancer-initiating cells induce T-cell apoptosis. The glioma-associated cancer-initiating cells supernatants were able to induce immune cell apoptosis in healthy donor PBMCs (Fig. 4A). All supernatants of the clonogenic cancer-initiating cells (n = 20) were able to increase immune cell apoptosis by 144 ± 29% (P = 0.0001) in healthy donor PBMCs (Table 1). Furthermore, when glioblastoma multiforme patients' PBMCs were coincubated with the respective patients' glioma-associated cancer-initiating cells, as predicted from the phenotypic expression of B7-H1 on glioma-associated cancer-initiating cells, both preapoptosis and apoptosis were induced in the immune cells (Fig. 4B), which was partially rescued by B7-H1 blockade (Fig. 4C), indicating that B7-H1 induces T-cell apoptosis by cell-to-cell contact with the cognate receptor on the T cells. Conditioned medium from normal human astrocytes and the U-87 glioma cell line did not induce T-cell apoptosis (Table 1). Activated immune cells also underwent apoptosis when cocultured with conditioned medium from glioma-associated cancer-initiating cells, indicating that activation did not protect immune cells from the apoptosis induced by conditioned medium from glioma-associated cancer-initiating cells (data not shown). This indicates that glioma-associated cancer-initiating cells can mediate immunosuppression by apoptotic elimination of immune cells, regardless of their activation state, likely by both secretion of product(s) and direct cell-to-cell contact.

Immunosuppressive properties of glioma-associated cancer-initiating cells are lost on alteration of their differentiation state. We next investigated whether the glioma-associated cancer-initiating cells could mediate immunosuppression after altering their state of differentiation. The glioma-associated cancer-initiating cells were exposed to differentiating medium (29) resulting in all of the glioma-associated cancer-initiating cell lines having alterations in cell morphology and increased expression of astrocytic (glial fibrillary acidic protein$^+$), neuronal (microtubule-associated protein 2$^+$), and oligodendroglial (galactosylceramidase$^+$) lineage markers (Fig. 1B). In addition, the expression level of CD133 was reduced after exposure to differentiation medium (mean, 5%; range, 0-11%; with one representative shown in Fig. 5A).

Conditioned media from the more differentiated glioma-associated cancer-initiating cells were then harvested, and the immunosuppressive properties were evaluated. We found that the inhibition of immune cell

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proliferation (n = 3) by conditioned medium from the glioma-associated cancer-initiating cells was reversed on altering differentiation (Fig. 5B). This was likely secondary to the fact that fewer FoxP3$^+$ Tregs were induced (Fig. 5C) and the T-cell apoptosis was diminished in the presence of conditioned medium from the more differentiated glioma-associated cancer-initiating cells (Fig. 5D). Furthermore, single-cell suspensions of glioma cells isolated from glioblastoma multiforme specimens that would contain the subpopulation of cancer-initiating cells were found to be immunosuppressive, which was lost on inducing differentiation (Table 1).

Galectin-3 secreted by glioma-associated cancer-initiating cells mediates induction of T-cell apoptosis. To ascertain the mechanism for the reduced T-cell apoptosis after differentiation of cancer-initiating cells, the supernatants from the cancer-initiating cells before and after differentiation and U-87 were assessed for Galectin-3 by ELISA. Before differentiation, the mean Galectin-3 produced by the cancer-initiating cells (n = 3) was 468 ± 77, 179 ± 10, and 139 ± 8 pg/10^6 cells/24 h, and after inducing an altered differentiated state, this was reduced to 18 ± 2, 41 ± 5, and 17 ± 2 pg/10^6 cells/24 h. U-87 cells did not produce Galectin-3. Next, recombinant Galectin-3 was added to the T-cell

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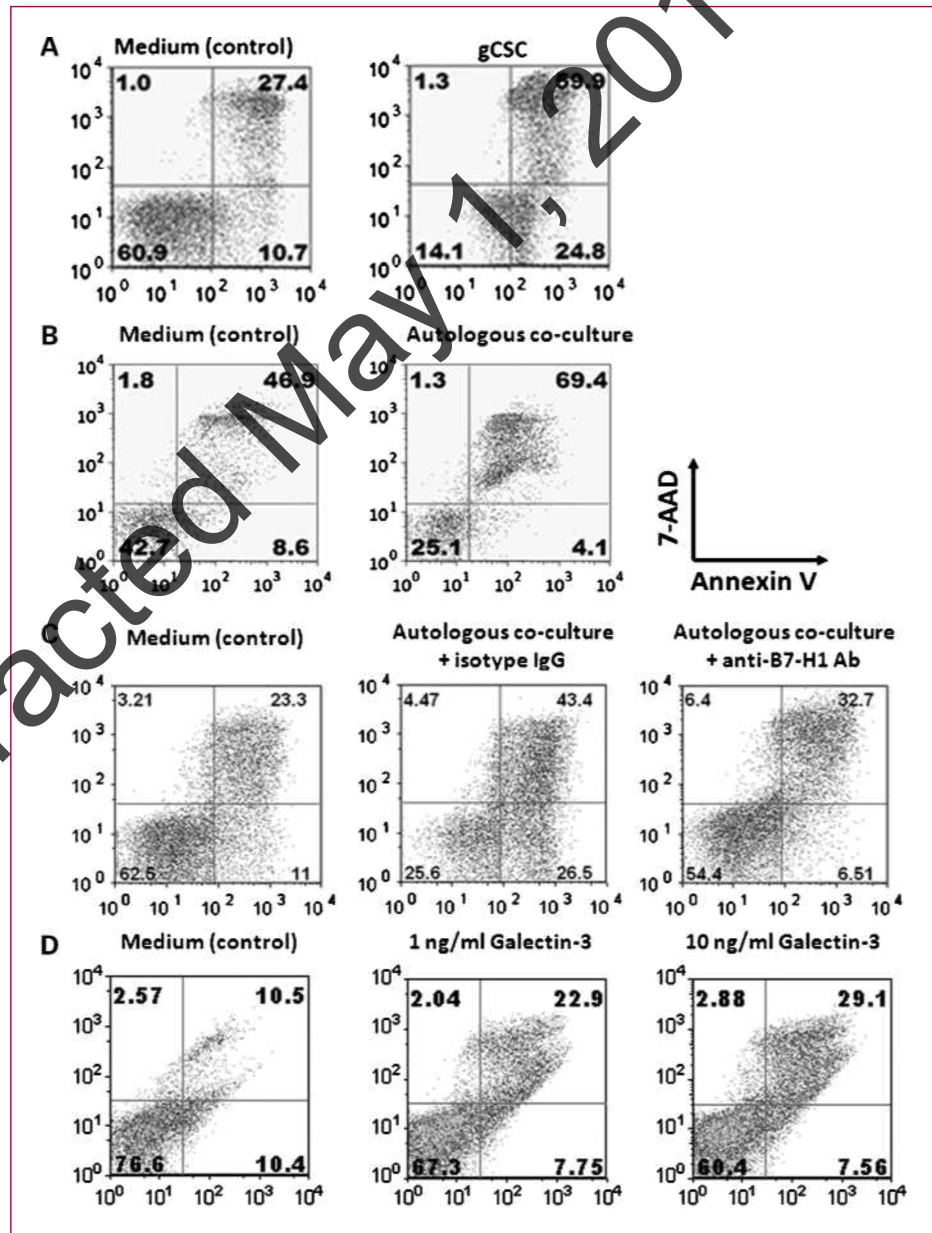

Fig. 4. Glioma-associated cancer-initiating cells trigger T-cell apoptosis. *A*, after culturing with the glioma-associated cancer-initiating cells supernatants, T cells were stimulated with anti-CD3/CD28 and stained with 7-amino-actinomycin D and Annexin V. Compared with medium alone (control), the glioma-associated cancer-initiating cells enhanced T-cell apoptosis. *B*, glioma-associated cancer-initiating cells induce T-cell apoptosis through cell-to-cell contact. The glioma-associated cancer-initiating cells were cocultured with autologous PBMCs at a 1:10 ratio, and an apoptotic assay was done after 3 days of culture. *C*, B7-H1 blockade in cell-to-cell contacting context reduces T-cell apoptosis. Both apoptotic and preapoptotic T-cell percentages decreased when addition of B7-H1 neutralizing antibody was added to culture conditions compared with the isotype control. One representative FACS plot is shown. Similar results were obtained for glioma-associated cancer-initiating cells and autologous PBMCs from two other patients. *D*, Galectin-3 can induce T-cell apoptosis in a dose-dependent manner within physiologic ranges produced by cancer-initiating cells supernatants.

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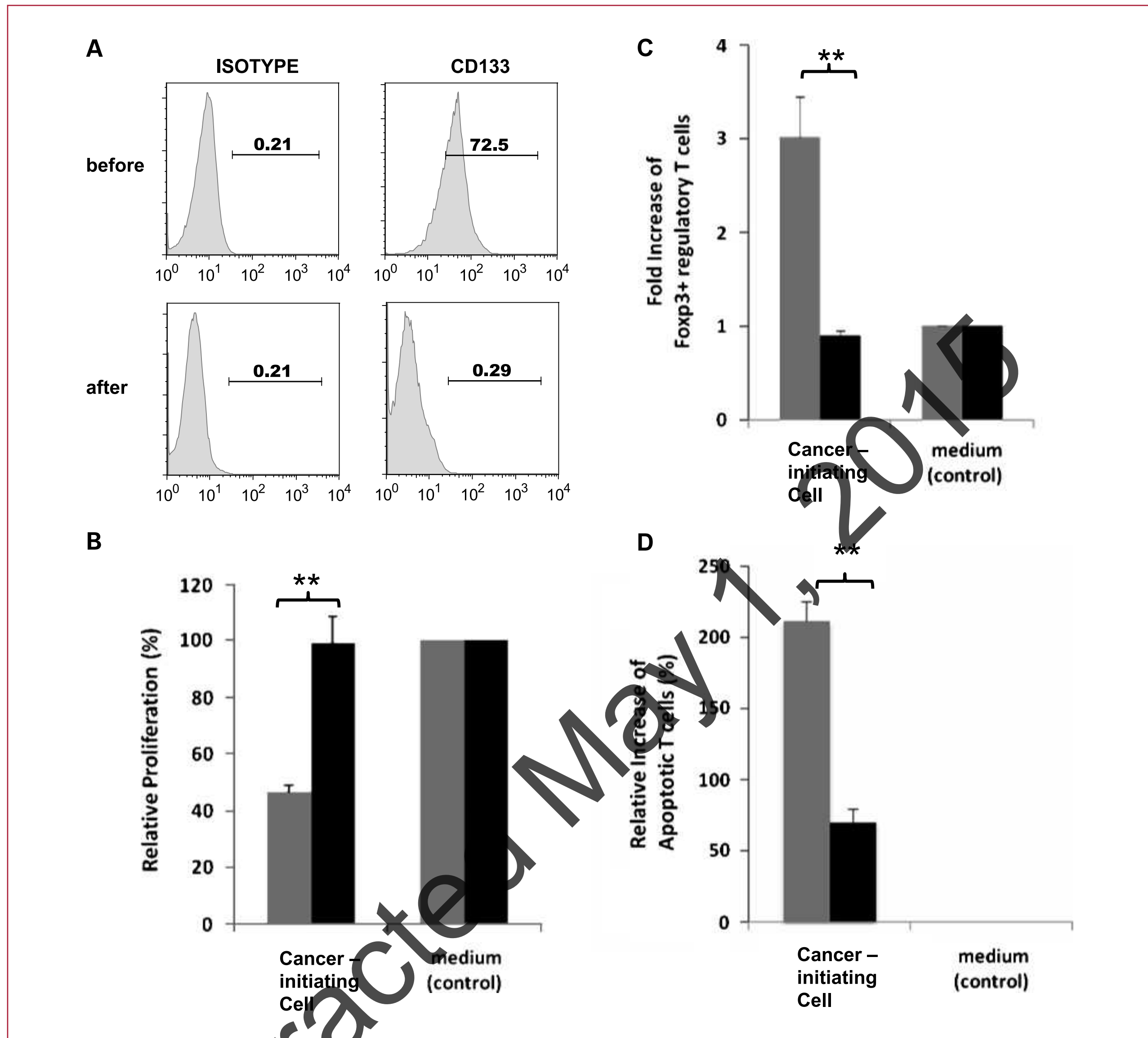

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Fig. 5. Glioma-associated cancer-initiating cells lose immunosuppressive properties on altering their state of differentiation. Glioma-associated cancer-initiating cells were cultured in neural stem cell medium or exposed to differentiating medium for two to three passages (5-7 days per passage). *A,* CD133 expression was reduced on the glioma-associated cancer-initiating cells on exposure to differentiation medium. *B,* glioma-associated cancer-initiating cells exposed to differentiating medium were less suppressive of T-cell proliferation. Healthy donor PBMCs were cocultured with supernatants from undifferentiated glioma-associated cancer-initiating cells or altered differentiated glioma-associated cancer-initiating cells in the presence of anti-CD3/CD28 stimulation, and cell proliferation was measured by Cell Counting Kit-8 after 4 days. T-cell proliferation in medium alone (control) was used as a baseline (100%), and the relative change in T-cell proliferation was plotted relative to this baseline. *C,* glioma-associated cancer-initiating cells exposed to differentiating medium do not induce FoxP3$^+$ Tregs. Cultured T cells on day 4 from *A* were stained for CD4 and FoxP3, and FACS data were converted into bar graphs showing fold change in the percentage of FoxP3$^+$ Tregs versus medium alone (control; set at baseline of 1). The results are averages from three independent experiments, with error bars showing SD. *D,* glioma-associated cancer-initiating cells exposed to differentiating medium reduces T-cell apoptosis. Cultured T cells on day 4 were analyzed for apoptosis, and the increase in apoptotic T cells was calculated as follows: percentage of apoptotic T cells in the presence of supernatants from the glioma-associated cancer-initiating cells minus percentage of apoptotic T cells in medium alone (control) divided by percentage of apoptotic T cells in medium alone. *B* to *D,* gray bars represent the results from conditioned medium from glioma-associated cancer-initiating cells; black bars represent the results from conditioned medium from differentiated glioma-associated cancer-initiating cells. This entire data set was obtained with similar results from two other patients. **, $P < 0.01$, significant difference between undifferentiated and differentiated glioma-associated cancer-initiating cells.

cultures to show that it is directly responsible for the immune cell apoptosis. At physiologic doses of Galectin-3 (1.5-3.2 ng/mL as determined by ELISA from the conditioned medium from cancer-initiating cells), there was dose response–induced T-cell apoptosis (Fig. 4D). Similar doses of Galectin-3 did not affect T-cell proliferation or induce FoxP3$^+$ Treg induction (data not shown), suggesting that multiple factors and mechanisms are involved

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in the immunosuppression mediated by glioma-associated cancer-initiating cells.

Discussion

The immunologic properties of human cancer-initiating cells have not been defined previously, and to our knowledge, this is the first study to show that these cells mediate many of the key features of immunosuppression and it explains a possible mechanism for resistance to immunotherapy in the clinic. To investigate the immune properties of glioma-associated cancer-initiating cells, we used two different experimental approaches. In the first approach, the supernatants from glioma-associated cancer-initiating cells were used in immunologic assays with T cells from healthy donors to determine the effects of glioma-associated cancer-initiating cells in the absence of preexisting T-cell immunosuppression while avoiding allogeneic responses that could confound the interpretation of the data. In the second approach, using glioblastoma multiforme patients' T cells and the respective patients' glioma-associated cancer-initiating cells, allogeneic interactions would not confound the data, allowing for analysis of direct cell-to-cell contact; however, preexisting immunosuppression in the patient T cells and secreted factor (s) from autologous cancer-initiating cells might dampen the extent of cell-to-cell contacting immunosuppression exerted by the glioma-associated cancer-initiating cells. Regardless of the experimental approaches, the data consistently showed that the glioma-associated cancer-initiating cells mediate immunosuppression by several redundant mechanisms.

In this study, although the glioma-associated cancer-initiating cells expressed MHC-I, they lacked MHC-II, CD40, and CD80, which would be anticipated to induce T-cell anergy (30) and this was confirmed in our functional assays of T-cell proliferation. Of note, this immune phenotype was homogeneous across the glioma-associated cancer-initiating cells from various glioblastoma multiforme patients regardless of CD133 expression. Although the immune phenotype of the cancer-initiating cells contribute, in part, to the T-cell immunosuppression, other mechanisms such as the expression of B7-H1 and the secretion of Galactin-3 and TGF-β also play significant roles. The glioma-associated cancer-initiating cells expressed the costimulatory inhibitory molecule B7-H1, which was shown previously to be a key factor mediating immune resistance in gliomas (19) and induces T-cell apoptosis (31). It was therefore not unexpected to find in the direct cell-to-cell contact experiments that the glioma-associated cancer-initiating cells induced T-cell apoptosis in the scenario of B7-H1 expression. We also found that a cell-secreted product(s) were mediating T-cell apoptosis; however, the exact factor eluded determination until we conducted a human antibody microarray to evaluate those factors whose expression was lost on inducing an altered differentiated state, which included the candidate Galectin-3. Soluble Galectin-3 has been shown to induce T-cell apoptosis (32), is constitutively expressed in glioma cell lines but not normal astrocytes or oligodendrocytes (33), and has been shown to enhance glioma proliferation and migration (34). Thus, the secretion of Galectin-3 appears to be a feature of nonterminally differentiated cells, which can be reduced, along with the immunosuppressive properties, on altering the differentiation state. Additionally, it was not entirely surprising to see that cancer-initiating cells could induce Tregs that were functionally active because the cancer-initiating cells did elaborate modest levels of TGF-β and this amount (10-100 pg/mL) of TGF-β that have been shown previously to be sufficient for Treg induction (35). Of note, the glioma-associated cancer-initiating cells did not elaborate nitric oxide as a possible mechanism for Treg induction (data not shown). Cumulatively, our data indicate that, in addition to the previously identified key role of glioma-associated cancer-initiating cells in radiation resistance (9) and chemotherapy resistance (3, 36, 37), glioma-associated cancer-initiating cells also contribute to immunosuppression.

Adult human stem cells have been shown to have potential clinical applications for a variety of degenerative diseases and for organ tissue replacement (38). As far as we are aware, we are the first group to propose altering the differentiation state of cancer-initiating cells as an approach to reverse immunosuppression that could be used in an immunomodulation/immunotherapeutic context. In this article, we showed that the cancer-initiating mediated immunosuppression can be reversed by altering the differentiation state. However, our findings pertaining to the reversal of immunosuppression on altering the differentiation state are in contrast to the studies of Le Blanc et al. who found that there was no change in alloreactive lymphocyte proliferative responses between differentiated and undifferentiated mesenchymal stem cells (39). The differences between allogeneic and autologous T-cell proliferation assays, the final differentiated cell phenotype, or a fundamental differences in the ability to reverse immunosuppression between these cell types may account for the differences in our findings compared with the previous study. Strategies that force glioma-associated cancer-initiating cells into a more differentiated phenotype (40) such as agents that block the signal transducer and activator of transcription pathway (41) may actually be promising agents as an immunotherapeutic approach (42). Induction of differentiation as a therapeutic strategy has been used with success in promyelocytic leukemia with all-*trans* retinoic acid (43) and many new compounds have been identified based on the isoquinoline sulfonamide scaffold that have been shown to induce differentiation in stem cells that may also be of clinical utility (44). Ultimately, the optimal agent would only induce differentiation in the cancer-initiating cells without effecting normal somatic stem cells. Further studies will

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be necessary to ascertain what type of differentiation agents would be optimal in combination with other immunotherapeutics.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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