

Antigen-Specific T-Cell Response from Dendritic Cell Vaccination Using Cancer Stem-Like Cell-Associated Antigens

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ABSTRACT

Glioblastoma multiforme (GBM) is the most aggressive primary brain tumor, with current treatment remaining palliative. Immunotherapies harness the body's own immune system to target cancers and could overcome the limitations of conventional treatments. One active immunotherapy strategy uses dendritic cell (DC)-based vaccination to initiate T-cell-mediated antitumor immunity. It has been proposed that cancer stem-like cells (CSCs) may play a key role in cancer initiation, progression, and resistance to current treatments. However, whether using human CSC antigens may improve the antitumor effect of DC vaccination against human cancer is unclear. In this study, we explored the suitability of CSCs as sources of antigens for DC vaccination against human GBM, with the aim of achieving CSC-targeting and enhanced antitumor immunity. We found

that CSCs express high levels of tumor-associated antigens as well as major histocompatibility complex molecules. Furthermore, DC vaccination using CSC antigens elicited antigen-specific T-cell responses against CSCs. DC vaccination-induced interferon- γ production is positively correlated with the number of antigen-specific T cells generated. Finally, using a 9L CSC brain tumor model, we demonstrate that vaccination with DCs loaded with 9L CSCs, but not daughter cells or conventionally cultured 9L cells, induced cytotoxic T lymphocytes (CTLs) against CSCs, and prolonged survival in animals bearing 9L CSC tumors. Understanding how immunization with CSCs generates superior antitumor immunity may accelerate development of CSC-specific immunotherapies and cancer vaccines. STEM CELLS 2009;27:1734–1740

Disclosure of potential conflicts of interest is found at the end of this article.

INTRODUCTION

Glioblastoma multiforme (GBM) is the most common and most aggressive type of primary brain tumor, accounting for 52% of all primary brain tumor cases. Current treatment of GBM remains palliative and includes surgery, radiotherapy, and chemotherapy [1, 2]. Immunotherapies harness the body's own immune system to target brain tumor and could overcome the limitations in conventional treatments [3–5]. One of the most promising strategies may be active immunotherapy using dendritic cell (DC)-based vaccination to initiate T-cell-mediated antitumor immunity [6–10]. In practice, vaccination strategies have often used DCs pulsed with tumor-derived whole lysates/peptides as modalities to present a broad range of tumor antigens to T cells *ex vivo* to stimulate effective antitumor T-cell immunity [8]. This process includes two stages: *in vitro* DC maturation and antigen loading and *in vivo* DC migration and antigen presentation in the draining

lymph node (DLN). Human DCs are commonly generated from peripheral blood-derived monocytes, followed by a differentiation step to produce immature DCs (iDCs). The iDCs undergo maturation and antigen loading steps to produce mature DCs [11]. Mature DCs loaded with tumor antigens are administered subcutaneously into patients. The goal is to generate *ex vivo* a population of antigen-loaded DCs that stimulates robust and long-lasting CD4⁺ and CD8⁺ T-cell responses in the patient with cancer.

Recent studies identifying cancer stem-like cells (CSCs) as brain tumor-initiating cells may have implications for modifying GBM treatments, including DC vaccination-based immunotherapy [8, 12–14]. Therapies targeting CSCs may prevent tumor recurrences seen after conventional radiation and chemotherapies. Furthermore, it is likely that certain stem cell markers expressed by CSCs may have distinct antigenicity and thus provide opportunities for enhanced immunotherapy. Some proteins expressed by CSCs are normally seen only in early development stages. Antibodies against the stem

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cell-associated antigen SOX2 was identified in a human patient [15]. CSC-associated proteins may be used for cancer vaccination. It was reported recently that vaccination using prostate stem cell antigen induced long-term protective immune response against prostate cancer without autoimmunity [16]. Even without identification of specific antigens, CSCs can be a useful source of tumor antigens in DC vaccination-based immunotherapy. Using a mouse GL261 glioma model, Pellegatta et al. demonstrated that vaccination with DCs loaded with glioma CSC antigens elicited robust antitumor T-cell immunity [17]. In this study, DC vaccination using CSC antigens cured up to 80% GL261 tumors, whereas DC vaccination using regular GL261 antigens cured none of the CSC-initiated tumors. However, whether using human CSC antigens may improve the antitumor effect of DC vaccination against human cancer is unclear.

In the current study, we investigate expression of tumor-associated antigens (TAAs) and major histocompatibility complex (MHC) molecules by GBM-derived CSCs. We report that CSCs express MHC I and increased levels of a range of TAAs. CSCs can be recognized by T cells generated after DCs were pulsed with CSC tumor antigens. DC vaccination-induced interferon (IFN)- γ production is positively correlated with the number of antigen-specific T cells generated. Finally, using a 9L CSC brain tumor model, we demonstrate that DC vaccination with 9L CSCs induced higher IFN- γ production than vaccination using parent cells and daughter cells and that DC vaccination with only 9L CSCs prolongs survival of tumor-bearing animals.

MATERIALS AND METHODS

Primary Culture of Glioblastoma-Derived Cancer Stem-Like Cells

Primary brain tumor spheres were cultured as previously described [14]. Briefly, brain tumor stem-like cells were grown in Dulbecco's modified Eagle's medium (DMEM)/F12 medium supplemented with B-27 (Invitrogen, Carlsbad, CA, <http://www.invitrogen.com>), 20 ng/ml of basic fibroblast growth factor, and 20 ng/ml of endothelial-derived growth factor (Peprotech, Rocky Hill, NJ, <http://www.peprotech.com>). Alternatively, dispersed brain tumor stem-like cells were grown on a laminin-coated surface in the same medium as described above. Primary human fetal neural stem cells were derived from primary cells obtained from Cambrex (East Rutherford, NJ, <http://www.cambrex.com>). GBM cell line and adherent primary glioma cells were cultured in DMEM/F-12 containing 10% fetal bovine serum. Some frozen primary GBM tissues were used to compare gene expression profiles of CSCs and their parental tumors.

Human Glioblastoma Cell Antigen Preparation

Human brain tumor cells were prepared following Institutional Review Board (IRB)-approved Standard Operating Procedure. Fresh tumor specimens were divided and processed under sterile conditions. Tumors were cleaned and minced in a dissection medium (Hanks' balanced salt solution, 0.36% glucose, 0.8 mM MgCl₂, 0.03 mg/ml catalase, 6.6 mg/l deferoxamine, 25 mg/l N-acetyl cysteine, 94 mg/L cysteine-Cl, 1.25 mg/L superoxide dismutase, 100 U/ml Fungi-Bact, 0.11 mg/ml sodium pyruvate, 10 mM HEPES). After digestion with trypsin-EDTA for 10 minutes at 37°C, cells were washed and passed through 100- μ m meshes. CSCs were isolated from primary tumor cells as described previously [8, 14]. At least 1 to 3 million cells in suspension were irradiated to prepare apoptotic CSCs as antigens. Tumor antigen protein concentrations were determined using Bio-Rad Protein Assay reagents (Hercules, CA, <http://www.bio-rad.com>).

Priming of Human DCs with Apoptotic CSCs

Human immature DCs were prepared from peripheral mononuclear blood cells (PBMCs). PBMCs obtained from a HLA-A2⁺ healthy donor were prepared by Ficoll/Paque (Invitrogen) density gradient centrifugation. Cells were seeded (1×10^7 cells/3 ml/well) into 6-well plates (Corning Costar Corp., Cambridge, MA, <http://www.corning.com/index.aspx>) in RPMI 1640 supplemented with 10% human AB serum, 2 mM L-glutamine, 10 mM HEPES, and antibiotics. After 2 hours of incubation at 37°C, adherent cells were used for DC generation as described. The nonadherent lymphocytes were stimulated with autologous DCs loaded with irradiated CSC line no. 66 (HLA-A2-positive, CD133-positive) at the ratio of 10:1 (the CSCs and DCs were cocultured overnight before they were seeded with lymphocytes). Interleukin-2 (IL-2; 300 IU/ml) was added to the cultures the next day and every 3 days thereafter. The lymphocytes were restimulated with DCs every week for up to 3 stimulations. The stimulated cells were tested for their ability to recognize antigen epitopes by incubating the stimulated cells with T2 cells pulsed with the epitopes as well as peptides derived from other tumor antigens and CD133-positive CSC lines. The stimulated cells (1×10^5) were incubated with 1×10^5 target cells for 24 hours, and the release of IFN- γ (pg/ml) was measured by commercial enzyme-linked immunosorbent assay (ELISA) kit (Endogen, Cambridge, MA, <http://www.piercenet.com>). The percentage of CD133-specific cytotoxic T lymphocytes (CTLs) in the stimulated cells was analyzed by tetramer technology.

Rat Glioma Cell Antigen Preparation and DC Culture

Rat 9L glioma cell antigens are prepared using a procedure similar to the one described above. All animal procedures were performed in strict accordance with the Institutional Animal Care and Use Committee guidelines at Cedars-Sinai Medical Center. For rat immature DCs isolation, F344 Fisher rats were euthanized and bone marrow cells were collected by flushing femurs and tibias with RPMI 1640 media. Bone marrow cells were incubated with IL-4 and granulocyte macrophage-colony stimulation factor (150 U/ml; Peprotech), with medium renewed every 2 days. After 7 days, cells were analyzed using fluorescence activated cell sorting (FACS) with antibodies against CD11c, CD14, CD80, CD86, and MHCII (obtained from BD Pharmingen, San Diego, <http://wwwbdbiosciences.com>). Immature DCs of 90%–100% purity were used for further studies. For rat DC stimulation, cells were stimulated by addition of lipopolysaccharide (50 μ g/ml, from *E coli* 055:B5; Sigma-Aldrich, St. Louis, <http://www.sigmaaldrich.com>) or tumor lysate (100 μ g/ml) for 1 day. Supernatant were taken after 1 day for further analysis.

Synthetic Peptides and HLA Typing

All of the peptides used in this study were synthesized by Macromolecular Resource (Fort Collins, CO, <http://www.macromolecular.colostate.edu>). The identity and purity of each of the peptides were confirmed by mass spectrometer and high-performance liquid chromatography analysis. Peptides were dissolved in dimethyl sulfoxide at 1 mM concentration for future use. GBM cells were stained with biotin-conjugated HLA-A2- or HLA-A1-specific monoclonal antibody (US Biological, Swampscott, MA, <http://www.usbio.net>) or biotin-conjugated isotype control antibody. After streptavidin-PerCP (BD Pharmingen) staining for 30 minutes, the mean fluorescence intensity of HLA-A2 staining was analyzed by flow cytometry.

Tetramer Staining

Various antigen-specific peptide tetramers (phycoerythrin-peptide-loaded HLA tetramer complexes) were synthesized [18, 19] and provided by Beckman Coulter (San Diego, <http://www.beckman-coulter.com>). Specific CTL clone CD8⁺ cells were resuspended at 10^5 cells/50 μ l fluorescence-activated cell-sorting buffer (phosphate buffer plus 1% inactivated fluorescence-activated cell-

sorting buffer). Cells were incubated with 1 μ l of tHLA for 30 minutes at room temperature, and incubation was then continued for 30 minutes at 4°C with anti-CD8 monoclonal antibody (Becton, Dickinson and Company, Franklin Lakes, NJ, <http://www.bd.com>). Cells were washed twice in 2 ml of cold fluorescence-activated cell-sorting buffer before analysis by fluorescence-activated cell sorting (Becton, Dickinson and Company).

T-Cell Stimulation and Cytotoxic T-Cell Assays

For human cells, CTL precursor frequency was determined to assess effective immunization to autologous tumor cells. PBMCs (1×10^6 cells/ml) were stimulated in 10% human AB serum with 1×10^6 /ml irradiated CSC-pulsed DCs, with recombinant human IL-2 (300 units/ml) added on day 2. Expanded CTLs from PBMCs were restimulated on day 11 with 150 μ g/ml tumor antigens for 2 hours. RNA was extracted and IFN- γ production was analyzed using ELISAs. Controls include DC alone, PBMC alone, and tumor antigens with PBMCs. Rat T-cell stimulation and CTL assays were performed in a similar fashion. FACS analysis of CD69 and MHC class II expression were performed for alloreactive T-cell activation index.

Real-time reverse-transcription PCR, ELISA, and FACS analysis

For quantitative polymerase chain reaction (PCR), total RNA was isolated from stimulated cells using Trizol (GIBCO Invitrogen, San Diego, CA, <http://www.invitrogen.com>) and transcribed using random hexamers. Cytokines and reference cDNA and quantified plasmid DNA standards were amplified using quantitative PCR primers or probes (Qiagen, Alameda, CA, <http://www1.qiagen.com>), with greater than a 1.5-fold increase in CD8-normalized IFN- γ production following vaccination indicating a positive response. ELISA analysis was performed to determine IFN- γ production using ELISA kits from R&D Systems Inc. (Minneapolis, <http://www.rndsystems.com>) following the manufacturer's instructions. DCs and PBMCs were rinsed in FACS buffer (phosphate-buffered saline with 1% FCS, 0.1% wt/vol sodium azide) and incubated with Fc Block (BD Biosciences, San Diego, <http://www.bdbiosciences.com>) for 20 minutes on ice, and stained with fluorescein isothiocyanate- or phycoerythrin-conjugated antibodies (CD86, CD69, MHC class II; from BD Biosciences) for 30 minutes on ice. After rinsing 3 times, cells were analyzed using a FACScan system (BD Biosciences).

DC Vaccination in a Rat Glioma Model

Adult F344 Fisher rats were anesthetized and placed in the stereotaxic frame. The skin was cut with a scalpel and the skull penetrated using a dental drill. A needle was placed 5-mm deep at the following coordinates from bregma (anterior-posterior: +1 mm; medial-lateral: 3 mm; dorsal-ventral: 5 mm). Duramater was punctured at the specific site of injection. Initial tumor inoculums were prepared using 25,000 luciferase-labeled 9L-gliosarcoma cells. For DC vaccination, at days 7, 14, and 21, animals received subcutaneous DC vaccinations into the flank, or via intracranial injection. Before vaccination, 50,000 freshly cultured immature dendritic cells were incubated with tumor antigens acid-eluted from tumor cells or saline control for 1 day.

Survival and Statistical Analysis

Data were analyzed with a SAS statistical software package (SAS Institute, Cary, NC, <http://www.sas.com>). Means of at least 3 independent experiments were reported with standard deviations. The estimated probability of survival was demonstrated using the Kaplan-Meier method. The Mantel Cox log-rank tests were used to compare curves between study and control groups. Any *P* values less than .05 were considered statistically significant.

RESULTS

Expression of Tumor-Associated Antigens in Glioblastoma-Derived Cancer Stem-Like Cells

We had previously isolated CSCs from human GBM and demonstrated their self-renewal capability and multipotent differentiation in vitro and tumor-initiating ability in vivo [8, 14]. Although these CSCs manifest different gene expression profiles and signal pathway activities [14], they share the expression of neural stem cell marker Nestin and the cell surface marker CD133 (Fig. 1A). Another cell surface marker LeX/CD15 was recently identified in murine neural stem cells [5]. Flow cytometry analysis indicated that most of the CD133-positive CSCs also express CD15 (Fig. 1B). The expression of CD133 and CD15 in the differentiated daughter cells, however, is greatly reduced (supporting information Fig. 1). To study whether CSCs express certain TAAs, we measured the expression levels of several GBM-associated tumor antigens in both conventional cultured adherent cells and corresponding CSCs. We found that both adherent cells that contain differentiated daughter cells and CSCs express EGFR, HER2, TRP2, MRP3, AIM2, SOX2, and IL13R α 2, but the expression levels were 2-fold to more than 200-fold higher in CSCs than those in adherent cells, with the exception of IL13R α 2, whose expression levels are comparable in both adherent cells and CSCs (Fig. 2). Some of these tumor antigens were shown in our previous studies to be expressed in human GBM and recognized by CTLs [7, 18, 19]. The higher expression of TAAs in CSCs suggested that CSCs may be a better source of tumor antigens in DC vaccination-based immunotherapy.

Expression of Class I MHC Molecules on Cell Surface of Cancer Stem-Like Cells

Tumor antigens must be presented with class I MHC molecules to be recognized by specific CTLs [20]. Tumor cells may down-regulate MHC expression through epigenetic modifications or other mechanisms to escape immune surveillance. To determine whether class I MHC molecules are expressed by GBM CSCs, the expression of MHC class I and class II was analyzed on 3 cancer stem cell lines and normal neural stem cells for comparison. As shown in Figure 3, all 3 CSCs clearly express class I MHC molecules on the cell surface, whereas its expression on neural stem cells (NSCs) is undetectable. Unexpectedly, a small population of each CSC line also expresses class II MHC molecules. Class II MHC expression is usually limited to the antigen-presenting cells (DCs, macrophages, and B cells). The significance of its expression in CSCs is unknown. We also detected similar expression of MHC molecules in adherent GBM tumor cells (data not shown). Therefore, CSCs express both TAAs and relevant MHC molecules that are necessary for CTL recognition and activation.

Vaccination with Dendritic Cells Loaded with Cancer Stem-Like Cell-Associated Antigens Elicits Antigen-Specific T-Cell Response

To determine the effect of DC vaccination using antigens enriched in CSCs, DCs isolated from PBMCs of an HLA-A2⁺ healthy donor were primed in vitro using autologous dendritic cells pulsed with irradiated apoptotic CSCs. These human mature DCs express costimulatory molecules CD80, CD86, and CD40 (supporting information Fig. 2). The expression patterns of costimulatory molecules by DCs after the culture with CSCs or their daughter cells are similar (supporting information Fig. 3). The lymphocytes were restimulated with DCs every week for up to 3 stimulations. The stimulated cells

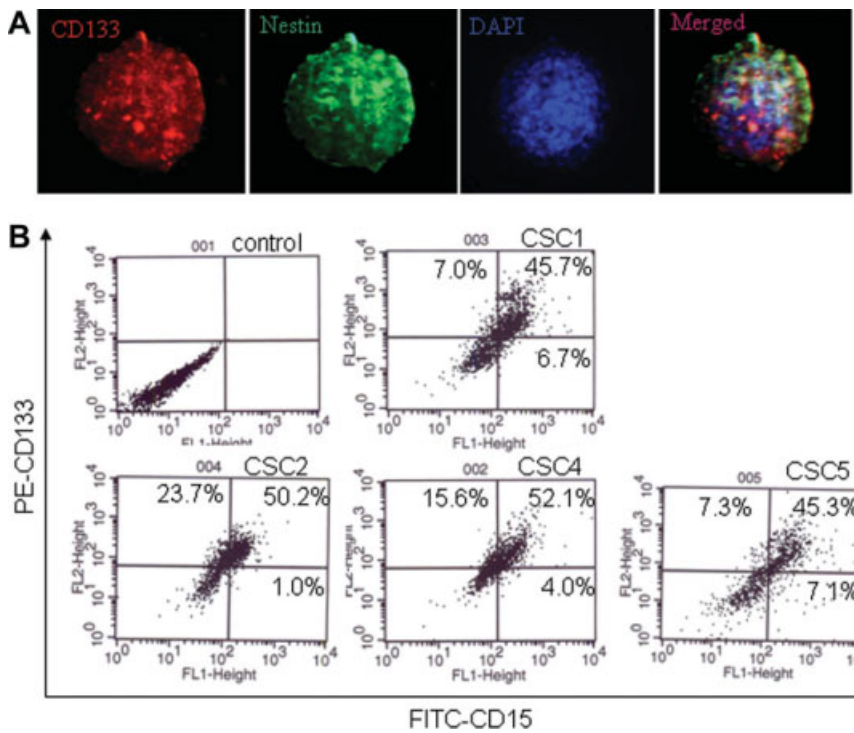


Figure 1. Glioblastoma-derived spheres are enriched in cells expressing cancer stem-like cell markers. (A): Glioblastoma-derived cancer stem-like cells express CD133 and Nestin. Primary cultured glioblastoma-derived spheres were processed for immunofluorescence staining using antibodies against human CD133 and Nestin. DAPI staining was used to reveal cell nuclei. (B): Glioblastoma-derived spheres are enriched in cells expressing both CD133 and CD15. Glioblastoma-derived spheres were processed into single-cell suspension before fluorescence activated cell sorting analysis using antibodies against human CD133 and CD15. Abbreviations: CSC, cancer stem-like cell; DAPI, 4',6-diamidino-2-phenylindole; dpi, dots per inch; FITC, fluorescein isothiocyanate; PE, phycoerythrin.

were then tested for their ability to recognize specific epitopes derived from TRP2, HER2, IL13R α 2, and SOX2 by incubating the stimulated cells with T2 cells pulsed with the epitopes. As shown in Figure 4A, tumor antigen-loaded DCs stimulated Th1 response and induced significant IFN- γ production. Next, we tested the effect of DC vaccination using 3 different CSCs and NSCs as antigens. Stimulation of PBMCs using each of the 3 CSC antigens induced significant T-cell responses as indicated by robust IFN- γ production in response to analysis stimulation (Fig. 4B). Although some antigens (such as EGFR) were shared between CSCs and NSCs, coculturing with NSCs did not induce immune response and IFN- γ production, consistent with the fact that NSCs do not express

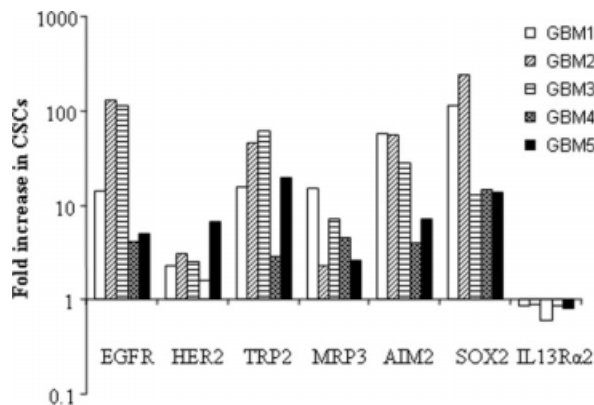


Figure 2. Elevated expression of tumor-associated antigens in human glioblastoma-derived stem-like cells. Representative tumor-associated antigen expression in glioblastoma multiform cancer stem-like cells. Cancer stem-like cells single-cell suspension was stained with specific monoclonal antibodies to each tumor-associated antigen and an isotype-matched control antibody, followed by fluorescence activated cell sorting analysis. Results are given as the ratio of antigen expression levels in CSCs to those in daughter cells. Abbreviations: CSCs, cancer stem-like cells; dpi, dots per inch; GBM1-5, glioblastoma multiform cancer stem-like cells 1-5.

MHC molecules. These data suggested that DC vaccination using apoptotic CSCs can elicit a strong specific T-cell response.

To determine whether the stimulated T cells contain antigen-specific CTLs, we performed tetramer analyses using antigen tetramers. Figure 4C shows that tumor antigen-specific CTLs were present in the in vitro-stimulated T cells. The percentages of antigen-specific CTLs vary from 0.3% to 1.2% (Fig. 4C). Clearly, optimization of peptide and tetramer designs and conditions may improve the detection of antigen-

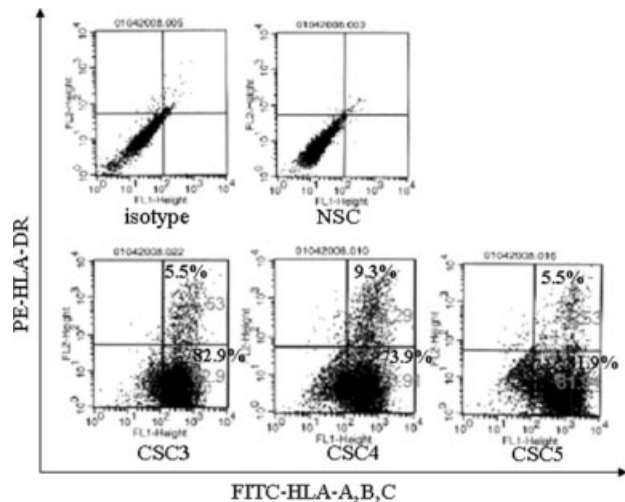


Figure 3. Glioblastoma-derived stem-like cells express class I MHC molecules on the cell surface. A subset of CSCs expresses both class I and class II MHC molecules. Single-cell suspension of neural stem cells and cancer stem-like cells was stained with specific antibody to class I MHC (HLA-A, B, C), class II MHC (HLA-DR) and an isotype-matched control antibody, followed by fluorescence activated cell sorting analysis. Results were representative of 3 independent experiments. Abbreviations: CSC3-5, cancer stem-like cells 3-5; dpi, dots per inch; FITC, fluorescein isothiocyanate; MHC, major histocompatibility complex; NSCs, neural stem cells; PE, phycoerythrin.

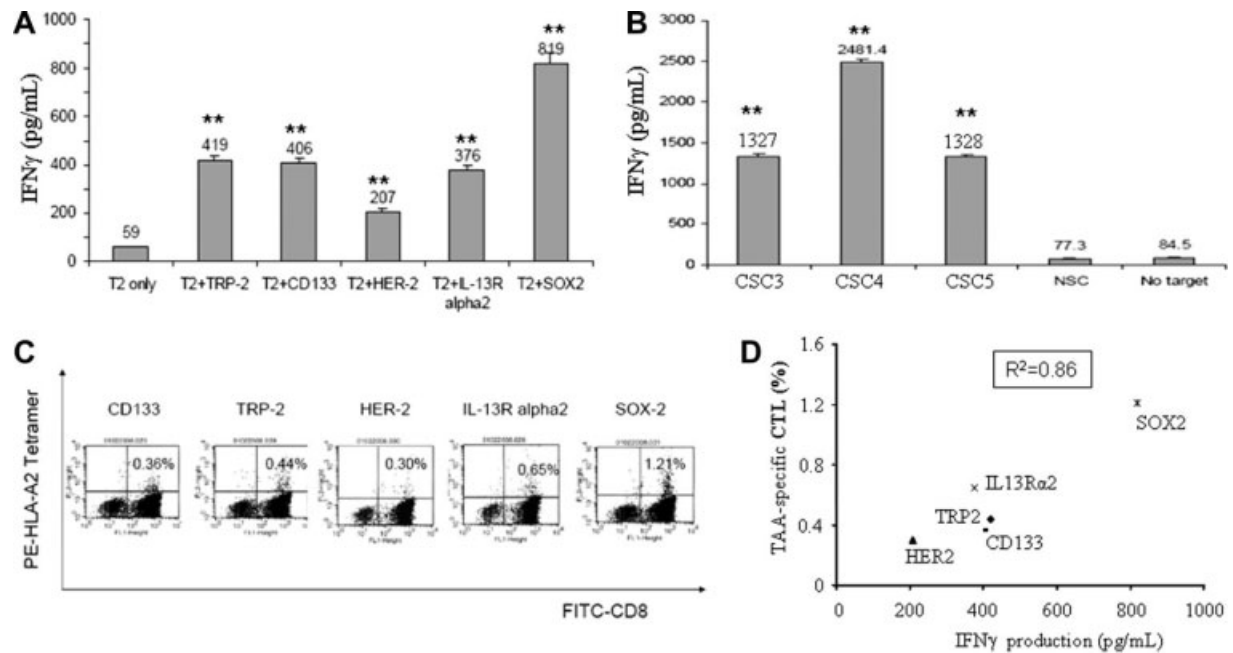


Figure 4. Dendritic cell vaccination using cancer stem-like cell antigens generates antigen-specific Th1 response. (A, B): IFN- γ release by antigen-specific CD8⁺ cytotoxic T lymphocytes recognizing tumor-associated antigens HER-2, TRP, CD133, IL13R α 2, and Sox2 (A) and IFN- γ release by CD8⁺ CTLs after vaccination with different CSCs (B). Clonal CTLs (1×10^5) were incubated with 1×10^5 HLA-matched tumor cells compared with CTLs incubated with 1×10^5 control cells. ** $p < .01$ (compared with control groups). (C): Assessment of tumor-associated antigen-specific T-cell clones by HLA/peptide tetramer staining. HER-2, TRP, CD133, IL13R α 2, and Sox2-specific CTL clones (1×10^5 cells) were stained with phycoerythrin-conjugated peptide/HLA tetramer at room temperature for 30 minutes. Cells were then incubated with antibody against CD8 for 30 minutes at 4°C. Cells were then examined by fluorescence-activated cell-sorting analysis using 10,000 events/sample. Generation of antigen-specific CTLs is positively correlated with Th1 response as indicated by IFN- γ production (coefficient $R^2 = 0.86$). Abbreviations: CSC3-5, cancer stem-like cells 3-5; dpi, dots per inch; CTL, cytotoxic T lymphocyte; FITC, fluorescein isothiocyanate; IFN- γ , interferon- γ ; NSC, neural stem cell; PE, phycoerythrin; TAA, tumor-associated antigen.

specific CTL generation. Finally, the percentage of antigen-specific CTLs is positively correlated with antigen-loaded DC-induced IFN- γ production ($R^2 = 0.86$) (Fig. 4D), suggesting that DC-induced IFN- γ production likely resulted from activation of antigen-specific CTLs.

To test whether there is any difference in tumor cell endocytosis levels by DCs after culture with CSCs and their daughter cells, which may contribute to differential TAA presentation and immune responses, we incubated DCs with PKH26-labeled apoptotic CSCs or their daughter cells and determined endocytosis frequencies in DCs. We found that there is no significant difference in endocytosis activities between DCs cultured with apoptotic CSCs and their daughter cells (supporting information Fig. 4), suggesting that superior immune responses after vaccination with CSCs rather than daughter cells are likely due to improved target cell recognition by antigen-specific CTLs.

DC Vaccination Using Cancer Stem-Like Cell-Associated Antigens Prolongs Survival in a 9L Brain Tumor Model

To investigate whether DC vaccination targeting CSCs may induce antitumor immunity in vivo and improve survival for tumor-bearing animals, we established a 9L CSC brain tumor model for DC vaccination studies. Previously, we isolated CSCs from 9L gliosarcoma cell line and demonstrated that 9L CSCs can initiate aggressive and chemoresistant brain tumors [21]. DCs were isolated from Fisher rat bone marrow, and were pulsed with media (as control) or acid-eluted tumor peptides from either 9L gliosarcoma cells grown in monolayer, 9L-CSCs, or 9L CSC daughter cells. Animals bearing intracranial 9L gliosarcoma were vaccinated subcutaneously on days 7, 14, and 21 after tumor implantations. Rats bearing 9L

gliosarcoma vaccinated with unpulsed DC vaccine all expired, with a median survival of 26.5 days. Rats vaccinated with DCs pulsed with 9L or 9L daughter cells also expired, with median survival dates of 32 and 29 days, respectively. In contrast, rats vaccinated with DC-9L CSCs had a median survival of 50 days when survival was monitored up to 70 days after inoculation, at which point 30% of the rats were still alive (Fig. 5). DC-9L CSC-vaccinated rats had slowly growing tumors that had increased infiltration of CD4⁺ T lymphocytes in brain sections (data not shown). The data demonstrate that DC immunotherapy could induce specific immune response targeting cancer stem-like cells and significantly prolong survival in a rat brain tumor model, suggesting that immunotherapy selectively targeting cancer stem cells could be a novel effective strategy to treat malignant glioma patients.

To determine whether the relative protective effect of 9L CSC DC vaccination on survival is due to tumor-specific immunity, we performed a CTL assay. IFN- γ mRNA levels in the CD8 cells were measured using real-time PCR. As shown in Table 1, restimulated splenocytes from rats treated with 9L CSC-pulsed DCs showed significantly higher level of IFN- γ mRNA in response to target than restimulated splenocytes from rats treated with 9L monolayer cells or 9L daughter cells. The higher IFN- γ mRNA response in the CSC-DC-vaccinated group is consistent with the higher survival rate observed in the same group, as shown in Figure 5.

DISCUSSION

Increasing evidence supports the notion that a small subpopulation of cancer stem-like cells is responsible for cancer

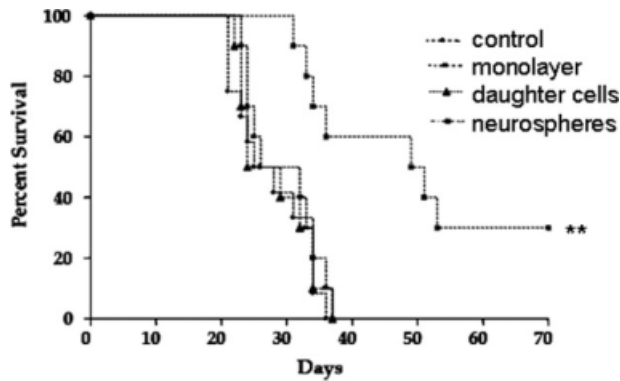


Figure 5. Therapeutic effect of neurosphere-pulsed dendritic cells (DCs) against intracranial 9L tumor in adult rats. Rats were injected intracranially with 25,000 9L glioma cells on day 0 and were vaccinated s.c. on days 7, 14, and 21 with different tumor antigen-pulsed dendritic cell vaccines: control (DC only), monolayer, daughter cells, and neurospheres (*n* = 10 for each group). Kaplan-Meier survival curve showed that rats treated with 9L neurosphere lysate-pulsed DCs have longer survival than the other groups (***p* = .0015). The surviving animals with cancer stem-like cell vaccination were sacrificed after 70 days and slowly growing brain tumors were detected. Abbreviation: dpi, dots per inch.

progression, therapy resistance, and relapse. To inhibit tumor recurrence more effectively, it would behoove any cancer therapy, including immunotherapy, to target the cancer stem cell. In this study, we build on our extensive experience of DC vaccination for human GBM patients, and explore the possibility of targeting CSCs in DC vaccination, using both human samples and a syngeneic animal brain tumor model. Our results demonstrate that GBM-derived CSCs express a range of TAAs and class I MHC molecules that is critical for immune recognition. The expression levels of some TAAs in CSCs are as high as more than 200-fold of the levels in daughter cells. Importantly, vaccination with DCs loaded with CSC antigens induced antigen-specific Th1 immune response. Finally, we tested DC vaccination using 9L CSC tumor antigens in a 9L CSCs brain tumor model and achieved robust antitumor T-cell immunity and a significant survival benefit.

Two recent studies using animal models demonstrated the potential of DC vaccination targeting CSCs in cancer immunotherapy [16, 22]. In their study of DC vaccination using GL261 neurospheres in a mouse brain tumor model, Pellegatta et al. found that immunization with DCs loaded with

GL261 neurospheres cured 60%--80% of animals with glioma, whereas vaccines of DCs loaded with adherent GL261 cells cured only 50% of GL261 tumors and none of the GL261 neurosphere-initiated tumors [17]. The authors also reported robust tumor infiltration by CD8⁺ and CD4⁺ T lymphocytes. Although there is no tumor antigen characterization, antigen presentation function, or other mechanistic data in this study, it indicated the distinct potential of CSCs in inducing antitumor immunity. In another recent study of prostate cancer, Pellegatta et al. vaccinated mice bearing progressing prostate cancer with prostate stem cell antigen and found induced MHC expression, cytokine production, lymphocyte infiltration, and long-term protection against prostate cancer [16]. Both cancer vaccination studies in murine models support the hypothesis that CSC-derived whole lysates or CSC-associated antigens may be superior to conventional tumor antigens in generating therapeutic antitumor effects. Consistent with these studies, our data on cancer immunization using 9L CSCs in a rat model indicated that vaccination with DCs loaded with only 9L CSCs antigens, but not the daughter cell antigens, induced CTL responses against CSCs and significantly extended survival of animals bearing 9L CSC tumors.

One key point from our study is that CSC-targeting DC vaccination appears to be superior not only in experimental murine models, but also in human brain tumors. Due to the known difference in cancer immunity between murine species and humans, it is important to investigate CSC-targeting DC vaccination in human cancer and compare the results in human cancer study to those in murine models. In our study, we took advantage of several well-characterized human GBM-derived CSCs [8, 14] and explored the possibility of DC vaccination using CSC antigens against human brain tumors. Significantly, we found that these CSCs highly express a range of known TAAs as well as MHC molecules. Immunization with apoptotic CSCs induced an antigen-specific Th1 response. These data suggest that CSCs may be better sources of antigens for cancer immunization than conventionally cultured tumor cells. It is to be seen whether this CSC-targeting vaccination will generate better antitumor clinical effects in human GBM patients. It is important that CSC-targeted DC vaccination should not lead to immune reaction to normal cells that may express common antigens. However, multiple mechanisms may exist that spare normal cells from such side effects. We found that NSCs had very low expression of cell surface MHC molecules. NSCs may also evade immune attack due to decreased expression of costimulatory

Table 1. IFN- γ production after immunization with 9L-cancer stem-like cell-pulsed dendritic cells

Samples	Mean Ct		Mean threshold difference (Δ Ct)	Fold change in normalized IFN- γ ($2^{\Delta\Delta Ct}$)
	CD8	IFN- γ		
Control				
No target	23.1	26.3	-3.2	1.32
Target	23.7	26.5	-2.8	
9L-monolayer				
No target	23.4	24.4	-1.0	1.15
Target	24.3	25.1	-0.8	
9L-daughter cells				
No target	24.8	28.8	-4.0	1.41
Target	22.9	26.4	-3.5	
9L-neurospheres				
No target	23.2	28.2	-5.0	5.66
Target	22.8	25.3	-2.5	

Splenocytes from tumor-bearing rats were restimulated with IL-2 for 11 days followed by re-exposure to a final 9L tumor cell target (9L monolayer cells, 9L neurospheres, or 9L).

Abbreviations: Ct, threshold cycle; IFN- γ , interferon- γ .

proteins [23]. We are in the process of initiating a clinical trial to study the safety and efficacy of DC vaccination using CSC antigens.

To date there is a very limited number of studies of CSC-targeting DC vaccination in animal models or in patients. And detailed immunological analysis data on the development of antitumor immunity after DC vaccination are not available. Questions remain regarding mechanisms underlying the apparent superior outcomes from CSC-targeting DC vaccination. For example, is there any difference between CSC antigens and conventional tumor lysates in promoting DC maturation and polarization, or in effector cell differentiation and memory T-cell generation *in vivo*? Although higher expression of TAAs in CSCs, as shown in our study, may be one factor contributing to the outcomes, it is likely other factors in addition to TAA expression levels also play a role. Finally, outcomes of DC vaccination may be improved when it is administered in combination with chemotherapy, radiotherapy, or other therapies [9, 24, 25].

SUMMARY

In summary, we explored the suitability of CSCs as sources of antigens for DC vaccination against human GBM, with the aims of achieving CSC-targeting and better antitumor immu-

nity. We found that CSCs express increased levels of TAAs as well MHC molecules. Furthermore, DC vaccination using CSC antigens elicited a potent antigen-specific Th1 response. Finally, we show that vaccination with DCs loaded with 9L CSCs, but not the daughter cells or conventionally cultured 9L cells, induced CTLs that recognized CSCs and prolonged survival of animals bearing 9L CSC tumors. Understanding how immunization with CSCs generates superior antitumor immunity may help develop novel and more effective cancer immunotherapies.

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DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

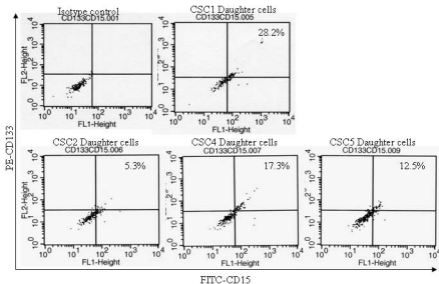
John Yu owned stock in, performed contract work for, served as an officer or board member for, and was employed by ImmunoCellular Therapeutics, Ltd.

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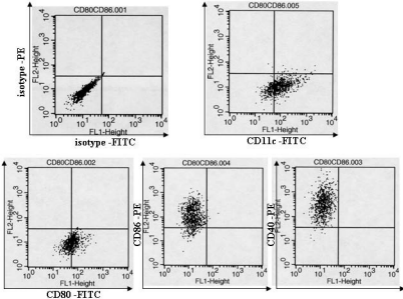
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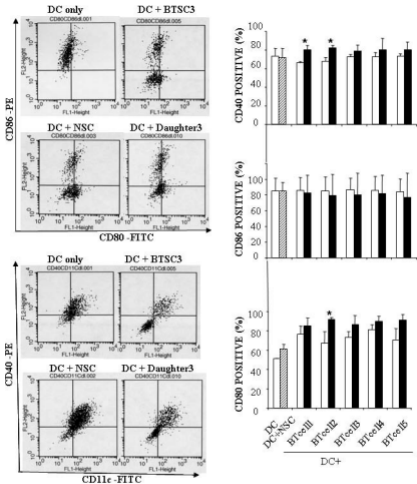
See www.StemCells.com for supporting information available online.



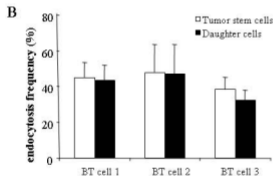
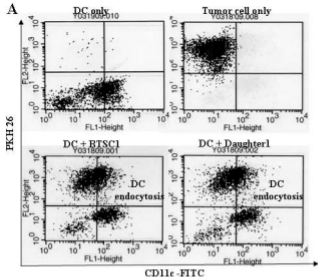
Supplemental Figure 1 Expression of neural stem cell markers CD133 and CD15 in differentiated brain tumor stem cells (daughter cells). For the preparation of CSC daughter cells, human CSCs were grown in differentiation medium (DMEM/F-12 containing 10% fetal bovine serum) for 10 days as monolayers. CSC daughter cells were analyzed for their expression of CD133 and CD15 as described in Figure 1. The percentages of CD133 and CD15 double positive cells (5.3-28.2%) decreased in daughter cells compared with those in CSCs (over 50%, see Figure 1).



Supplemental Figure 2 Characterization of human dendritic cells after maturation, showing the expression of lineage marker CD11c and co-stimulator signal molecules CD80, CD86, and CD40. Human dendritic cells were grown for seven days in the presence of recombinant human granulocyte macrophage colony-stimulating factor (GM-CSF, 800 units/ml; clinical grade; Immunex) and recombinant human interleukin (IL)-4 (500 units/ml; R&D Systems) and treated with human tumor necrosis factor (TNF)- α (50ng/ml) for two days. Cells were then harvest for FACS analysis with following antibodies or isotype controls (1:50 dilution): anti-human CD80-FITC (BD Bioscience), anti-CD11c-FITC, anti-human CD86-PE, anti-human CD40-PE (eBioscience). The CD11c- CD80-, CD86-, and CD40-positive dendritic cells were 71%, 50%, 89%, and 97%, respectively. Data were representative of three separate experiments.



Supplemental Figure 3 Effects of apoptotic tumor cell incubation on human dendritic cell (DC) co-stimulator expression. Human DC (1×10^6 /ml) were incubated with medium, neural stem cells, brain tumor stem cells or their daughter cells (2:1 ratio) for 20 h. Cells were then harvest for FACS analysis with antibodies against human CD40, CD86, and CD80. Left panels, representative FACS data including results from DCs with one pair of brain tumor cells and controls. Right panels, quantitative analysis of co-stimulator molecule expression by DCs after incubation with tumor cells (BTcell1-5) or neural stem cells (open bar, DC only or with tumor stem cells; closed bar, DC with corresponding daughter cells). Data were presented as mean /S.D. of three separate experiments. *, $p < 0.05$ (between tumor stem cell and daughter cell pairs).



Supplemental Figure 4 Comparison of endocytosis frequencies of human DCs during incubation with brain tumor stem cells or their daughter cells. Tumor cells (BT cell 1-3) were labeled with red fluorescent dye PKH26 (Sigma) following manufacturer's manual. Human DC (2×10^6 /ml) were incubated with PKH26-labeled apoptotic brain tumor stem cells or their daughter cells (3:1 ratio) for 20 h. Cells were then harvest for FACS analysis after staining for CD11c. A. Representative FACS data including results from DCs with one pair of brain tumor cells and controls. Note the distinct populations of DCs and tumor cells. B, quantitative analysis of endocytosis frequencies by DCs after incubation with tumor stem cells (open bar) or their daughter cells (closed bar). Endocytosis frequencies were calculated as percentage of PKH26 and CD11c double positive cells in total CD11c positive cells. Data were presented as mean /S.D. of three separate experiments. There is no statistically significant difference between tumor stem cells and daughter cell pairs.