Neurospheres Enriched in Cancer Stem–Like Cells Are Highly Effective in Eliciting a Dendritic Cell–Mediated Immune Response against Malignant Gliomas

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Abstract

Cancer stem–like cells (CSC) could be a novel target for cancer therapy, including dendritic cell (DC) immunotherapy. To address this, we developed experiments aimed at DC targeting of neurospheres (NS) from GL261 glioma cells because neurospheres can be enriched in CSC. We obtained murine neurospheres by growing GL261 cells in epidermal growth factor/basic fibroblast growth factor without serum. GL261-NS recapitulated important features of glioblastoma CSC and expressed higher levels of radial glia stem cell markers than GL261 cells growing under standard conditions (GL261 adherent cells, GL261-AC), as assessed by DNA microarray and real-time PCR. GL261-NS brain gliomas were highly infiltrating and more rapidly lethal than GL261-AC, as evidenced by survival analysis ($P < 0.0001$), magnetic resonance imaging and histology. DC from the bone marrow of syngeneic mice were then used for immunotherapy of GL261-NS and GL261-AC tumors. Strikingly, DC loaded with GL261-NS (DC-NS) cured 80% and 60% of GL261-AC and GL261-NS tumors, respectively ($P < 0.0001$), whereas DC-AC cured only 50% of GL261-AC tumors ($P = 0.0022$) and none of the GL261-NS tumors. GL261-NS expressed higher levels of MHC and costimulatory molecules (CD80 and CD86) than GL261-AC; the JAM assay indicated that DC-NS splenocytes had higher lytic activity than DC-AC splenocytes on both GL261-NS and GL261-AC, and immunohistochemistry showed that DC-NS vaccination was associated with robust tumor infiltration by CD8+ and CD4+ T lymphocytes. These findings suggest that DC targeting of CSC provides a higher level of protection against GL261 gliomas, a finding with potential implications for the design of clinical trials based on DC vaccination. (Cancer Res 2006; 66(21): 10247-52)

Introduction

Malignant gliomas (MG) include glioblastoma multiforme (GBM), the most frequent and aggressive of primary brain tumors (1). The best standard of care for GBM, including surgery followed by radiotherapy and chemotherapy with temozolomide, is associated with a median overall survival of 14.6 months following diagnosis (2). Considerable efforts have been spent in designing new treatments for GBM and MG. Dendritic cell (DC)–based immunotherapy is becoming the target of increasing expectations because of the very encouraging results obtained in preclinical experiments in MG (3). More recently, phase I and II clinical trials have shown that DC vaccination in relapsing GBM is safe, but data on efficacy (mostly evaluated as overall survival) are not yet satisfactory (4). Among the different approaches to be considered for improvement, the definition of more precise targets seems of paramount importance. Clearly, an increased understanding of the molecular pathways underlying GBM development is required for the identification of these novel targets.

Evidence is growing that in GBM, as in other solid tumors, cell hierarchies exist, so that not all the neoplastic cells are endowed with the same tumorigenic potential (5). The fraction of tumor-initiating cells has been operationally defined as cancer stem–like cells (CSC). Using the same growth factors employed for neural stem cells, neurospheres (NS) were also obtained from GBM. These GBM-NS seem to be enriched in CSC, differently from GBM cells that, under standard conditions, grow as adherent cells (AC; refs. 6, 7). If CSC have a critical role in tumor re-initiation, their targeting by DC may offer a greater potential to control or abolish tumor growth. We have addressed this in an experimental system based on neurospheres obtained from the murine MG cell line, GL261.

Materials and Methods

Cell cultures. The murine glioma cell line, GL261 (here defined as GL261 adherent cells, GL261-AC), was grown in DMEM (EuroClone, Wetherby, United Kingdom), 20% fetal bovine serum, L-glutamine, and penicillin/streptomycin. GL261 neurospheres (GL261-NS) were obtained by growing GL261-AC in DMEM/F12 (Life Technologies, Gaithersburg, MD), supplemented with penicillin/streptomycin sulfate, B-27 (Life Technologies), human recombinant fibroblast growth factor 2 (FGF-2; 20 ng/mL; Peprotech, Rocky Hill, NJ), epidermal growth factor (EGF; 20 ng/mL; Chemicon, Germany), and heparin (Sigma-Aldrich, St. Louis, MO).

DC vaccinations. Murine DC were prepared from the bone marrow of C57BL6/J.EGFP transgenic mice (H-2^b) and cultured in Iscove's medium (Sigma-Aldrich), in the presence of 5 ng/mL recombinant granulocyte macrophage colony-stimulating factor and 5 ng/mL recombinant interleukin 4 (Li StarFISH, Milan, Italy). The tumor lysate obtained by sonication of 1×10^7 GL261-AC or neurospheres was prepared as described by Ashley et al. (8). Cell surface markers of DC were evaluated by flow cytometry before and after pulsing with monoclonal antibodies (BD PharMingen, San Diego, CA): CD11c, CD80, CD86, MHC class I, and MHC class II.

A total of 70 C57BL6/N mice (Charles River, Calco, Italy) were treated using six different combinations. GL261-AC tumors versus PBS 1× ($n = 14$), DC-NS

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

This report is dedicated to the memory of Giovanni Scropetta (Giò).

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(n = 10), and DC-AC (n = 12). GL261-NS tumors versus PBS 1× (n = 14), DC-NS (n = 8) and DC-AC (n = 12). Mice were injected intracranially with 1×10^5 GL261-AC or GL261-NS on day 0 and treated with s.c. injections of 1×10^6 DC pulsed with lysates from GL261-AC or GL261-NS (DC-AC and DC-NS, respectively) on days 7, 14, and 21.

Histology and immunohistochemistry. A representative number of brain and lymph nodes from each group were embedded in ornithine carbamoyltransferase (OCT) and snap-frozen in cold isopenthane in liquid nitrogen. Five-micrometer sections were taken on a cryostat and analyzed using anti-CD8 (Novocastra Laboratories, United Kingdom) and anti-Ki67 antibodies (BD PharMingen). See Supplementary Information for remaining methods.

Cytotoxicity assay. Cytotoxicity was investigated *in vitro* using the JAM test. Splenocytes from vaccinated and control mice were cocultured for 5 days with GL261 treated with mitomycin C at 40:1 E/T ratio, in the presence of recombinant human interleukin 2 (10 units/mL). GL261-AC and GL261-NS labeled overnight with 1 μCi of [^{3}H]thymidine were seeded with prestimulated splenocytes from control and vaccinated mice at different E/T ratios.

Statistical analysis. Survival estimates and median survivals were analyzed using the Kaplan-Meier method. Student's t test was used for calculating the significance of data. Statistical significance was determined at the <0.05 level. Methods for magnetic resonance imaging (MRI), DNA microarray analysis, and real-time PCR are reported in the Supplementary Information.

Results

Neurospheres from MG cell line GL261 are similar to CSCs. To investigate if CSCs could be obtained from GL261 cells, we cultured them in epidermal growth factor/basic fibroblast growth factor without serum. After 5 days, neurospheres were visible in the medium and were proliferating steadily. Their growth increased exponentially regardless of passage number, whereas GL261-AC growth tended to plateau (Fig. 1*A*). Cloning analysis showed that GL261-NS have a higher potential for clone formation that GL261-AC: clonal frequency was 24% and 7% of the total cells plated, respectively.

Using real-time PCR, we found that expression of the stem cell marker, nestin, was 4.5-fold higher in neurospheres than in AC (Fig. S1*A*). Flow cytometry confirmed that a large fraction of GL261-NS expressed nestin: when switched to a differentiation medium, the fraction of nestin-positive cells decreased from 47.4% to 27.3%. The expression of neural differentiation markers (GFAP, βIII tubulin, and CNPase) increased considerably (Fig. 1*B* and Fig. S1*B*); 3.6% of cells coexpressed neuronal and astrocytic markers. Interestingly, GL261-AC did not express these markers under standard conditions of culture and failed to grow under differentiating conditions (data not shown).

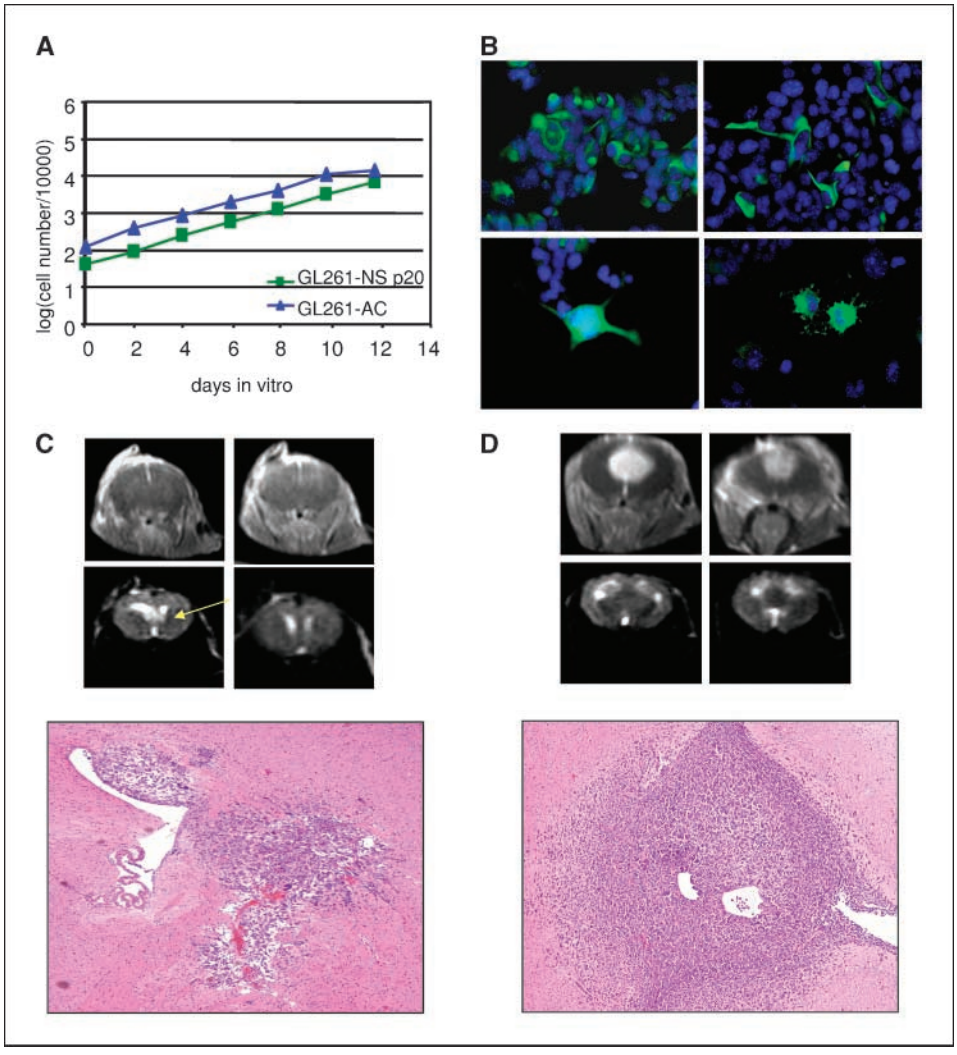

Figure 1. Neurospheres from GL261 glioma cells (GL261-NS) share relevant features with CSCs from GBM. *A,* proliferation kinetics of GL261-NS and GL261-AC (from passages 20 to 26) shows that GL261-NS cultured in EGF/bFGF proliferate at a constant rate, whereas GL261-AC cultured in serum show initial proliferation followed by a plateau phase (see Supplementary Information for methods). *B,* GL261-NS cultured for 6 days with EGF/bFGF express nestin, as confirmed in this cytospin preparation (*top left*). Under differentiating conditions, GL261-NS adhere to the plastic and express glial or neuronal markers: the astrocytic marker GFAP (*green, top right*), the neuronal marker βIII tubulin (*green, bottom left*), and the oligodendroglial marker CNPase (*green, bottom right*). *C* and *D,* MRI, 1.5 T images on a frontal plane. *Top,* T1 after contrast medium injection. *Bottom,* T2. *C,* a GL261-AC tumor on day 15. No modifications on T1 images. On T2 images, although a partial volume effect cannot be excluded, a hyperintensity is visible that seems slightly more diffuse towards the midline (*arrow*). The right frontal ventricular horn is enlarged. H&E staining of the GL261-AC tumor shown by MRI (magnification, ×4; *bottom*). *D,* a GL261-NS tumor on day 15. A large lesion is shown both in T1 images as an homogeneous strongly enhancing rounded mass, and in T2 images, as a rounded hypointensity with strong mass effect towards lateral ventricles. The hypointensity is suggestive for high cellularity of the tumor. H&E staining of the GL261-NS tumor shown by MRI (magnification, ×4; *bottom*).

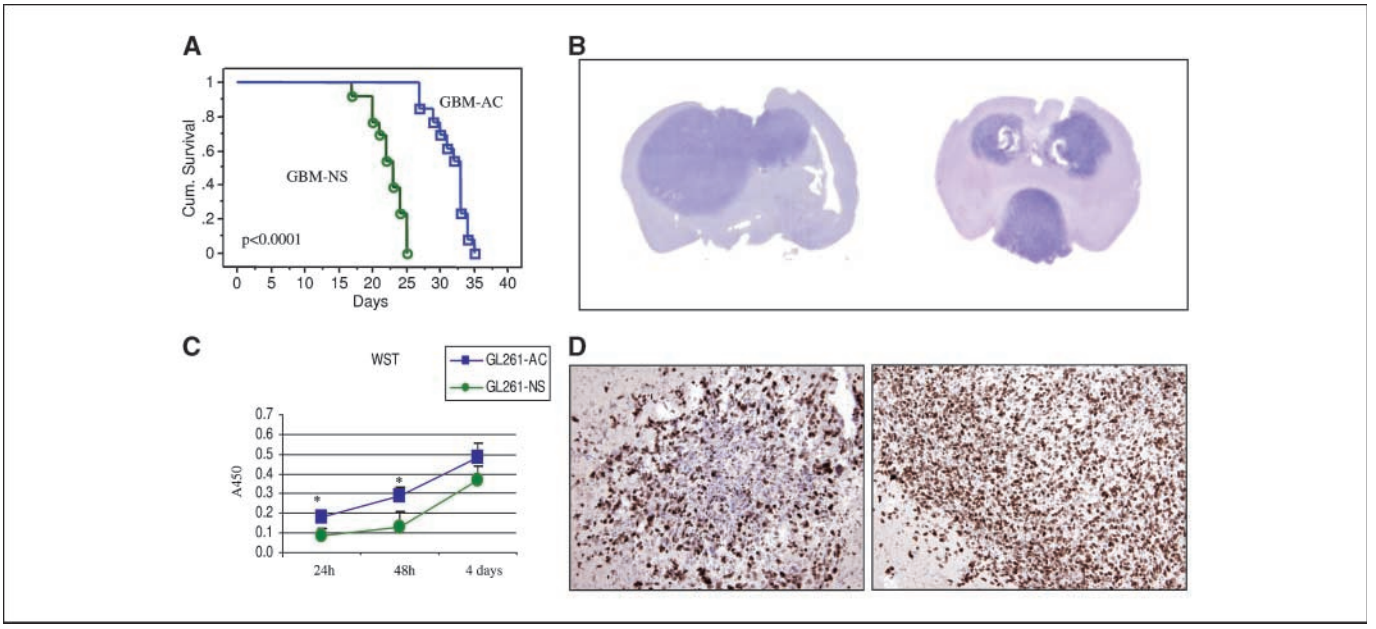

Figure 2. GL261-NS form highly aggressive gliomas in C57BL6/N mice. *A,* Kaplan-Meier survival analysis shows that GL261-NS ($n = 14$) are significantly more aggressive than GL261-AC ($n = 14$; $P < 0.0001$). *B,* morphology of a GL261-AC that was lethal on day 31 after injection of tumor cells (*left*). Morphology of a GL261-NS that was lethal 17 days after injection of tumor cells (*right*). *C,* cell proliferation was evaluated by a colorimetric method using the WST reagent. WST analysis, evaluated 24 hours, 48 hours, and 4 days after plating cells indicates that *in vitro* GL261-NS proliferate less than GL261-AC (24 hours, $P = 0.009$; 48 hours, $P = 0.006$). *D, left,* Ki-67 staining of a GL261-AC tumor from a mouse sacrificed on day 15 after injection of tumor cells (magnification, ×10); *right,* Ki-67 staining of a GL261-NS tumor from a mouse sacrificed on day 15 after injection of tumor cells (magnification, ×10).

To compare their *in vivo* growth, GL261-NS and GL261-AC cells were injected into the striatum of syngeneic C57BL6/N mice. We looked for tumor formation at different time points. On day 7, GL261-AC tumors were small and localized in the vicinity of the injection site (Fig. S2*A*), whereas GL261-NS tumors aggressively infiltrated the brain parenchyma, were attracted towards subependymal spaces, infiltrated the ependyma, and reached the ventricles (Fig. S2*B*). The MRI analysis done on day 10, however, showed little differences in mouse brains injected with GL261-AC or GL261-NS (Fig. S2*C* and *D*). The different patterns of growth of GL261-AC and GL261-NS gliomas could be fully appreciated on day 15 (Fig. 1). On MRI, a small lesion derived from GL261-AC is visible on T2 images in the left hemisphere (Fig. 1*C*); the lesion derived from GL261-NS, on the contrary, is much larger and located between the two ventricles (Fig. 1*D*). Histology done on the same mice, sacrificed the day after MRI, confirmed these findings (Fig. 1*C* and *D*). Kaplan-Meier analysis for survival is shown in Fig. 2*A* and confirms that GL261-NS are significantly more aggressive than GL261-AC gliomas. The median survival of mice injected with GL261-NS was 22.5 ± 3.2 days, whereas that of mice injected with GL261-AC was 32.5 ± 5.3 days ($P < 0.0001$). Histology exemplifying the different growth patterns of GL261-AC and GL261-NS at the time of death is shown in Fig. 2*B*. Although the WST assay for proliferation showed that *in vitro* GL261-NS grow slower than GL261-AC (Fig. 2*C*), Ki-67 staining suggested that *in vivo* GL261-AC have a lower proliferation rate than GL261-NS (Fig. 2*D*).

Expression profiling identifies a radial glia signature in GL261-NS. Our results show that GL261-NS recapitulate relevant features of the CSC paradigm. *In vitro*, they grow at a consistent rate and can differentiate in the three neural phenotypes. *In vivo*, they create highly infiltrating MGs. To further characterize the GL261 model, we defined gene expression profiles of GL261-NS and GL261-AC, using GeneChip Mouse Genome 430A 2.0 Array, representing ~14,000 well-characterized mouse genes. Both types of cell lines were studied in duplicate.[5] After background correction and normalization, the four resulting data sets were subjected to the limit fold change model analysis producing a selection of 774 probe sets (Table S1).

Transcripts were then grouped because of their involvement in interaction with the extracellular matrix, cell proliferation, cytoskeleton and process outgrowth, signal transduction, relationship with the immune system, neurogenesis, and angiogenesis (Table S2). We focused validation experiments on genes indicating the relationship of GL261-NS with neural stem cells. In particular, five genes up-regulated in GL261-NS seemed to be coherently related to radial glia: *Reelin*, a positive regulator of radial glia (9); *Olig2* and *aldolase C* (or *zebrin II*; ref. 10); *Fabp7*, or brain lipid-binding protein, and *Slc1a3*, encoding the glutamate transporter GLAST (11). Increased expression of these five genes in GL261-NS was confirmed by real-time PCR (Table S3).

Also of interest is the observation that data on genes regulating angiogenesis showed consistently that GL261-NS are more proangiogenic than GL261-AC. Increased expression of Vegfa, Hif1α, Flt1, and angiopoietin 2 were associated with decreased expression of thrombospondin 1. Results on vascular endothelial growth factor (VEGF) were validated by ELISA, showing that GL261-NS, and not GL261-AC, produce high levels of VEGF that increase with passaging (Fig. S3*A*), and by immunohistochemistry, showing that VEGF expression is higher in GL261-NS than in GL261-AC tumors (Fig. S3*B-D*).

DC loading with GL261-NS but not GL261-AC lysates provides effective protection against GL261 gliomas. The identification of subgroups of GL261 cells with different behavior

[5] Data available at http://www.ebi.ac.uk/arrayexpress/ with accession E-MEXP-718.

in vitro and *in vivo* may imply that such subgroups are differentially targeted by therapies. We addressed this possibility by DC-based immunotherapy, loading DC derived from the bone marrow of enhanced green fluorescent protein transgenic mice with lysates from GL261-NS or GL261-AC cells (DC-NS and DC-AC, respectively): DC were injected s.c. 1 week after intracranial injection of GL261-NS or GL261-AC. Evidence for DC maturation after pulsing was obtained by flow cytometry (Fig. S4*A*). DC capacity for migration was confirmed by immunohistochemical analysis with anti-GFP antibody, detecting several positive cells in the axillary lymph node homolateral to DC injection (Fig. S4*B*).

The survival analysis showed that DC-NS provide significant protection against both GL261-NS and GL261-AC tumors (Fig. 3*A, left*). Strikingly, DC-AC only afforded a partial protection against GL261-AC tumors, but failed completely to mount an immune response against GL261-NS tumors (Fig. 3*A, right*). Using flow cytometry, we then examined the expression levels of MHCI (H2b) and MHCII (Ia) and of costimulatory molecules, CD80 and CD86, in GL261-NS and GL261-AC. Interestingly, all these markers were highly expressed in GL261-NS and less in GL261-AC (Fig. 3*C*), suggesting that GL261-NS are more immunogenic than GL261-AC. In agreement with this, direct injection of GL261-NS lysates was more effective than injection of GL261-AC lysates in protecting against GL261-AC tumors (Fig. S5*A-C*).

CD8+ T-lymphocytes are important effectors of DC-mediated immune responses against GL261-NS gliomas. To evaluate the ability of DC pulsed with tumor lysate to prime a CTL response against autologous tumors *in vivo*, mice were injected intracranially with GL261-AC ($n = 2$) or GL261-NS ($n = 2$) and vaccinated 7 days later. Ten days after vaccination, mice were killed and splenocytes restimulated with autologous tumor cells and tested 5 days later for lytic activity against either GL261-AC or GL261-NS. Splenocytes from mice immunized with DC-NS induced a specific lytic activity against GL261-AC and GL261-NS, whereas mice immunized with DC-AC revealed a lytic activity against GL261-AC only (Fig. 3*B*).

To characterize immune responses in the brain and define lymphocyte subsets involved in the generation of antitumor activity, we studied intratumoral leukocyte infiltration in the mice evaluated by JAM test. Histologic analysis revealed a more

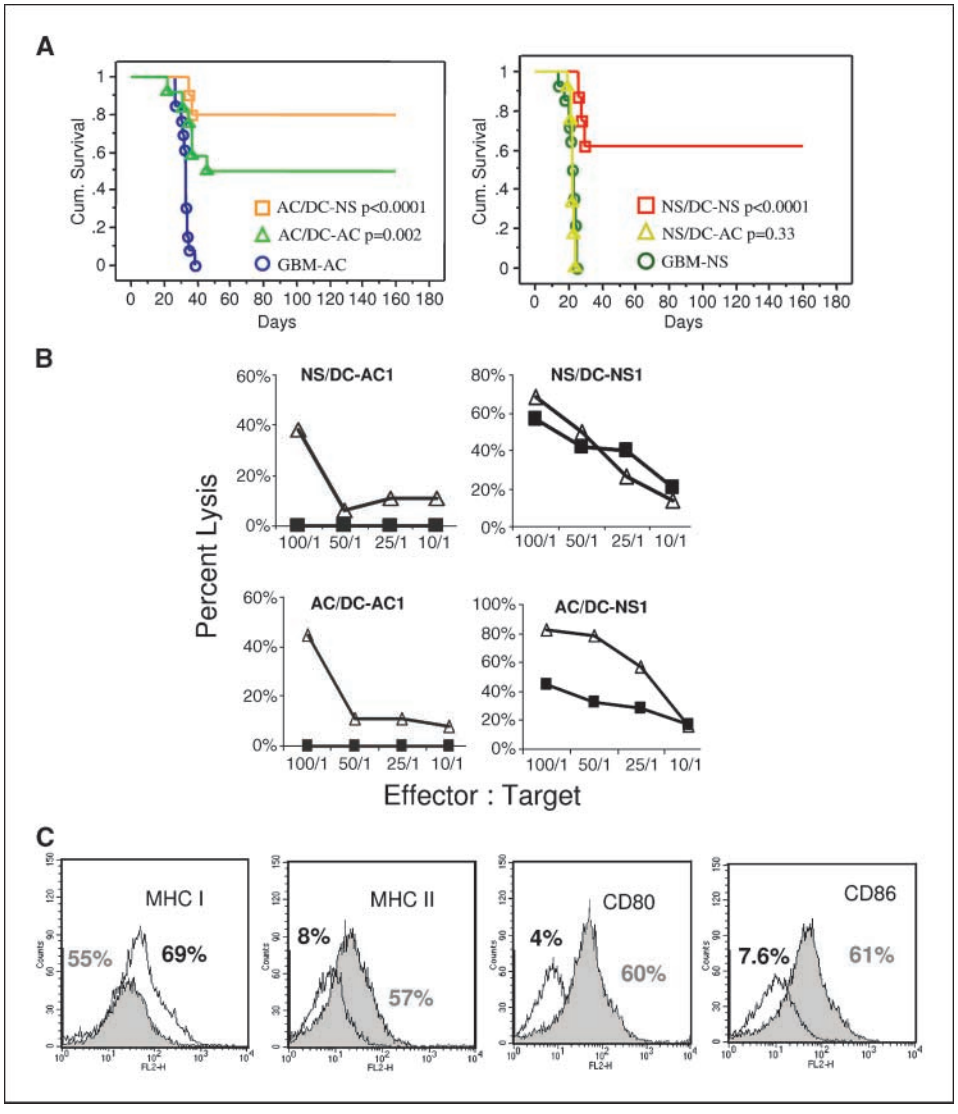

Figure 3. DC loaded with GL261-NS lysates are highly effective against GL261 gliomas. *A, left,* Kaplan-Meier survival curves: 36 C57BL6/N mice injected intracranially with 1×10^5 GL261-AC on day 0 were treated s.c. on days 7, 14, and 21 with PBS (GL261-AC, $n = 14$), DC-NS ($n = 10$, $P < 0.0001$ versus GL261-AC), DC-AC ($n = 12$, $P = 0.0022$ versus GL261-AC). *Right,* Kaplan-Meier survival curves: 34 C57BL6/N mice injected intracranially with 1×10^5 GL261-NS cells on day 0 were treated s.c. on days 7, 14, and 21 with PBS (GL261-NS, $n = 14$), DC-NS ($n = 8$, $P = 0.0001$ versus GL261-NS), and DC-AC ($n = 12$, $P = 0.33$ versus GL261-NS). *B,* induction of specific lytic activity against tumor cells by immunization with DC pulsed with tumor lysates. In the JAM assay, splenocytes derived from each mouse were tested against GL261-AC (△) and GL261-NS (■). Results refer to one representative animal for each group. *C,* immunologic characterization by flow cytometry of GL261-AC (*white areas*) and GL261-NS (*gray areas*). Overlay diagrams show the expression of the relevant antigens on GL261-NS versus GL261-AC. The figure indicates the up-regulation of MHC II and costimulatory molecules on GL261-NS. The results are representative of four different experiments. Data on immune cells infiltrating the tumor in these mice and in the other mice tested are summarized in Supplementary Table S4.

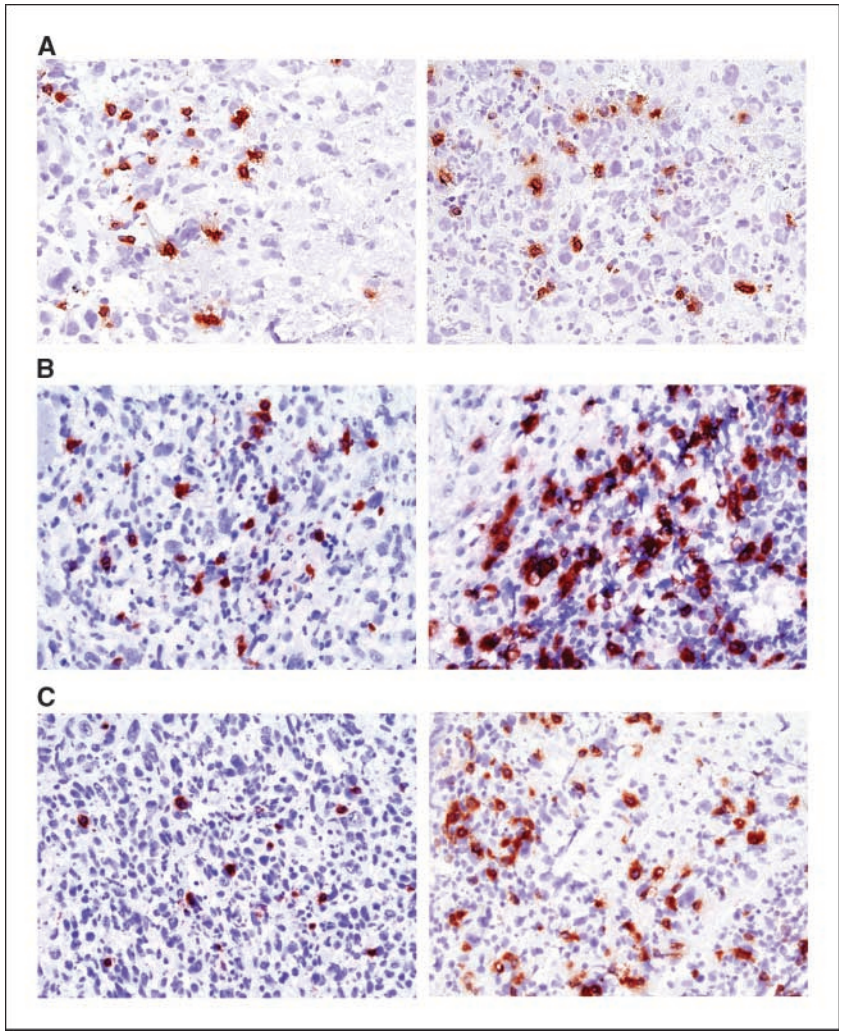

Figure 4. CD8-positive T lymphocytes are critical effectors of DC-mediated immune rejection of GL261 gliomas. *A,* scattered CD8-positive cells are present in the brain of untreated mice injected with Gl261-AC (*left*) and GL261-NS (*right*). *B,* brain gliomas from GL261-AC in mice treated by DC-AC (*left*) or by DC-NS (*right*) show increased infiltration of CD8-positive T lymphocytes. *C,* brain gliomas from GL261-NS in mice treated by DC-AC (*left*) or by DC-NS (*right*) show a further increase in the infiltration of CD8-positive T lymphocytes (magnification, ×20).

abundant infiltration of CD3/CD8/CD4-positive cells in mice treated with DC-NS compared to animals immunized with DC-AC and to controls treated with PBS (Fig. 4; Figs. S6 and S7; Table S4). Analysis by flow cytometry of inflammatory and effector cells after Percoll gradient confirmed the pivotal role of CD8+ cells in DC-primed immune responses (Fig. S8).

Discussion

The results we obtained by culturing GL261 cells using conditions appropriate for neural stem cell expansion imply that even after years of passaging under differentiating conditions, this glioma cell line may retain the capacity for dedifferentiation to a stem-like phenotype. Similar findings were obtained by Kondo et al. on a rat GBM cell line (12). Kondo and Raff also showed that chromatin remodeling may allow us to move backward along the differentiation passages, going from a neural stem cell to a fully differentiated glial cell (13). Our data point to a relationships between expression profiles of GL261-NS and radial glia. Radial glial cells not only serve as neural progenitors during central nervous system development but also give rise to adult subventricular zone stem cells (14).

FGF is an important regulator of S-type radial glial cells in the subventricular zone (15): thus, growth factors for GL261-NS, including FGF, may facilitate the (re-)acquisition of the phenotype of slow-dividing, S-type radial glial cells, including FABP7 expression and GFAP lack of expression. Recent data have suggested that CSC in ependymomas originate from radial glia (16) and we are presently investigating whether our observations are confirmed in human GBM.

We have found that DC targeting of GL261-NS provides more efficient protection against GL261 tumors than targeting of GL261-AC. The GL261 tumors we have originated are of two types: GL261-AC grow *in vivo* like other glioma cell lines, forming large noninfiltrating tumors bearing little resemblance to primary MG (7). GL261-NS, on the contrary, are highly infiltrating and rapidly lethal and share more similarities to MGs. Expression profiling, *in vivo* growth, and their potential for neural differentiation confer to GL261-NS important characteristics of CSCs. Recent data have suggested that CSC are at the core of malignant growth, being the only cells equipped for *in vivo* tumorigenesis (5). Thus, our results can be partly explained by considering that DC targeting of GL261-NS depletes the tumor of its hierarchically highest and most relevant population: CSCs.

We also found that GL261-NS, but not GL261-AC, express high levels of MHC class II and costimulatory molecules, CD80 and CD86. Expression of MHC class II antigens is significantly down-regulated

in GBM cells on early passages compared with the primary tumor: the expression levels may increase partially only after extensive passaging under standard conditions (DMEM and 10% fetal bovine serum; ref. 17). The expression levels of CD80 and CD86 could also increase after passaging but are very low in primary tumors (17). Normal neurospheres, on the other hand, express MHC II molecules but are weakly immunogenic because of the low expression levels of costimulatory molecules (18). Therefore, it seems that GL261-NS have a peculiar set of expression of molecules highly relevant for immune recognition. It is conceivable that EGF plays a role in determining such expression: in breast cancer cells, for example, EGF increases HLA I and HLA II expression (19). Furthermore, EGF may increase nuclear factor κB expression (20) and nuclear factor κB, in turn, significantly up-regulates CD80 and CD86 expression (21, 22). Thus, culture conditions of GL261-NS may be responsible for this result and justify the increased efficacy of DC loaded with GL261-NS extracts.

To translate our observations to the clinical setting, we need to assess how representative of human MG the GL261 model. We previously found that human GBM-NS are more tumorigenic *in vivo*, and more representative of genetic alterations of the primary tumor (6). We confirmed these observations in a larger number of gliomas[6] and data consistent with these findings were reported recently (7). When analyzed in five of these tumors, HLA-DR and costimulatory molecules were always more expressed in neurospheres than in AC (data not shown).

If these two features of the GBM-NS subpopulation, higher representativity of tumor-initiating cells and higher immunogenicity, will be confirmed in a larger series of observations, their translational perspective could be relevant. GBM cells from relatively small amounts of tumor tissue could be amplified *in vitro* as neurospheres and used for treating the relapse, usually taking place 6 months or more after the first surgery.

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