

Fusion of Glioblastoma Tumor Antigens to Herpes Simplex Virus-1 Glycoprotein D Enhances Secondary Adaptive Immune Responses in a DNA Vaccine Strategy

Rios WM¹, De Molfetta JB², Brandão IT¹, Masson AP¹, Peripato R², Silva ID², Rodrigues RF¹, Arnoldi F¹, De Souza PRM³, Diniz MDO⁴, Ferreira LCDS⁴ and Silva CL^{1*}

¹The Center for Tuberculosis Research, Department of Biochemistry and Immunology, School of Medicine of Ribeirao Preto, University of Sao Paulo, Brazil

²Farmacore Biotechnology, Brazil

³University Federal of Sergipe, Brazil

⁴Department of Microbiology, Biomedical Sciences Institute, University of Sao Paulo, Brazil

*Corresponding author: Silva CL, Departamento de Bioquímica e Imunologia, Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo, Avenida Bandeirantes, 3900, CEP 14049-900, Ribeirão Preto/SP, Brazil, Tel: +551633153086; E-mail: clsilva@fmrp.usp.br

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Abstract

Glioblastoma multiforme (GBM) is a recurrent and fatal cancer. EGFRvIII, MAGE-3 and GLEA-2 are antigens that are found in this highly heterogeneous tumor and that are absent in normal tissue. Usually, conventional GMB treatments do not prevent recurrence, reinforcing the need for new therapeutic strategies. Vaccination can be an alternative GMB therapy capable to induce long-lasting and specific immune responses to tumors antigens but requires the activation of strong cellular responses. Fusion of tumor antigens with microbial-derived proteins is a rather simple approach that can enhance the immunogenicity of vaccines, particularly, DNA vaccines. In this study, we constructed DNA vaccines encoding GBM tumor antigens fused to glycoprotein D from herpes simplex virus-1 and evaluated their immunogenicity in C57BL/6 mice. The tumor antigens were correctly expressed by the DNA vaccines and induced cell mediated immune responses under experimental conditions. The vaccines encoding antigens genetically fused with gD induced higher cellular immune responses, associated with IFN- γ and IL-10 production, than vaccines encoding non-fused GMB antigens. Therefore, DNA vaccinations induced a Th1-biased immune response. We concluded that the strategy could be an effective immunotherapeutic approach for GBM.

Keywords: Glioblastoma antigens; Immunotherapy; DNA vaccine; Glycoprotein D; IFN- γ ; IL-10

Abbreviations

gD: glycoprotein D; EGFRvIII: Variant of the type I epidermal growth factor receptor; MAGE: Melanoma antigen gene; GLEA: Glioma-expressed antigen; TMZ, temozolomide; BBB, blood-brain barrier; KLH, keyhole limpet hemocyanin; CT: Cancer testis; HVEM: Herpes virus entry mediator; BTLA: B and T-lymphocyte attenuator; MDSCs: Myeloid-derived suppressor cells.

Introduction

Glioblastoma multiforme (GBM) (WHO grade IV astrocytoma) is the most common and malignant primary cancer in the central nervous system (CNS) [1-3]. Despite the conventional and aggressive treatment, including maximal surgical resection followed by temozolomide (TMZ) and radiation, the prognosis for patients with GBM remains poor, with a median survival of less than 15 months [4,5]. One great challenge in developing GBM therapies is the complexity of the GBM microenvironment [6]. The typical characteristics of this disease include uncontrolled cellular proliferation, diffuse infiltration, necrotic tendency, significant angiogenesis, apoptosis resistance and tumor heterogeneity [7]. Furthermore, patients with GBM display profound immunosuppression [8], which seems, partially, triggered by the

cancer cells, as reported by Wei and colleagues [9]. Therefore, effective therapies must not only be directly cytotoxic to a molecularly diverse tumor cell population but also must overcome the pro-tumorigenic properties of the GBM microenvironment.

Thus, studies have actively focused on testing new therapeutic approaches, including immunotherapy. Active immunotherapy, represented by therapeutic anti-cancer vaccines, is a primarily attractive approach to fight different forms of cancer since it provides the advantage of cellular specificity and generation of long-term immune surveillance to recurrent cancer cells [2]. Furthermore, the change in the concept that the CNS is immunologically privileged has generated enthusiasm for a potential role for immunotherapy in GBM. The primary line of active immunological defense for the brain is composed of specialized resident cells called microglia [10]. These cells are activated when they migrate toward inflammatory areas, where they obtain phagocytic properties and produce cytokines and chemokines, allowing the recruitment of other immune cells [10]. Additionally, macrophages and dendritic cells (DCs) can act as powerful antigen-presenting cells (APCs) in the CNS [11,12]. Some studies have shown that antigens originating within the CNS are drained in the cerebrospinal fluid to nasal and cervical lymph nodes where adaptive immune reactions are initiated [13,14]. Moreover, subpopulations of activated T cells expressing a particular phenotype 4/7 integrin overexpression exhibit tropism for the brain and cross the blood-brain barrier (BBB) [15]. Taken together, these findings represent the evolution of the knowledge regarding the interactions

between the CNS and the immune system and strengthen the idea that immunotherapy can be an effective weapon in GBM treatment.

Selection of good antigens remains a considerable challenge both for passive and active immunotherapies for cancer. Some antigens associated with GBM have been described. EGFRvIII, the most common and well-characterized variant of the type I epidermal growth factor receptor (EGFR), was first identified in primary human GBM [16,17].

This variant form is constitutively active, and its expression confers tumorigenic properties to the cell [18,19]. Clinical trials have been performed with a 14-mer-derived peptide EGFRvIII, PEPvIII [16], conjugated to KLH (keyhole limpet hemocyanin), delivered like peptide-based vaccines or pulsed with DCs [20-23]. The results showed that this strategy could induce antigen-specific cellular and humoral immune responses and increase patient median survival [22,23]. The MAGE-3 (melanoma antigen gene) antigen has also been described in GBM. Chi et al. showed that MAGE-3 mRNA was present in 33% of the GBM cases but it is absent in normal tissue [24]. This antigen, which is a member of the MAGE family, is characterized as a cancer-testis (CT) antigen that is expressed in normal testis and several tumors [25-27]. Other researchers have identified MAGE-3 peptides that can be recognized by CD4+ T cells of individuals with or without cancer [28,29], indicating that this antigen may be the target of an immune response. The GLEA-2 (glioma-expressed antigen 2) antigen, identified in a GBM cDNA library [30], is immunogenic and considered a putative GBM antigen. Fischer and collaborators showed that 43% of the studied GBM patients presented antibodies against this antigen [30]. Furthermore, patients undergoing radiotherapy presented increased frequencies of anti-GLEA-2 antibody [31].

Although all these characteristics indicate that these antigens can be potential targets for GBM immunotherapy, the immunogenicity of cancer antigens is usually limited. Therefore, these antigens must be combined with other substances to achieve the desired response. The genetic fusion of glycoprotein D (gD) from herpes simplex virus-1 (HSV-1) to tumor antigens encoded by DNA vaccines has been used as an alternative to increase the immunogenicity of proteins, generating sustained tumor regression [32-34]. HSV-1 gD is a structural component of the virus envelope that is essential for virus entry into host cells [35]. This protein recognizes at least three receptors, namely, HVEM (herpes virus entry mediator), nectin 1 or 2 and 3-O sulfated heparan sulfate (3-O-S-HS), and triggers fusion of the viral envelope with the cell membrane [36]. HVEM was primarily described as a gD ligand; however, it also interacts with the LIGHT molecule, BTLA (B and T-lymphocyte attenuator) and with CD160 [37-42]. The HVEM-BTLA or HVEM-CD160 interaction elicits inhibitory signals to T cells, which inhibit the lymphocyte proliferation [39-42], and gD can interrupt this HVEM-BTLA/CD160 inhibitory pathway [32]. Furthermore, the gD-cell interaction induces DC phenotype maturation and activates NF- κ B and type I IFN secretion [43,44]. These cytokines activate IL-12 production by DCs, subsequently promoting the link between immediate and adaptive immune responses and activating efficient Th1 responses [43,44]. Thus, we show in this study that DNA vaccines expressing GBM tumor antigens as fusion proteins within HSV-1 gD induced marked cellular immune responses. The DNA vaccines induced specific memory T cells that produced IFN- γ and IL-10 after a secondary stimulus [43,44]. Thus, this DNA vaccine strategy represents a promising alternative for GBM immunotherapy combined with conventional therapy.

Materials and methods

Construction of DNA vaccines

The coding sequences were provided from human proteins named EGFRvIII, MAGE and GLEA. The sequencing data were submitted to the GenBank databases under accession numbers NM_005228 (EGFRvIII), NM_005362.3 (MAGE), and AF258787 (GLEA). These sequences without their stop codons were combined to the gD nucleotide sequence in silico. The fusion was made between two unique sites (PvuII and ApaI) that are present in gD [45]. The gDEGFRvIII, gDMAGE, gDGLEA fragments were constructed by Epoch Biolabs (Missouri, TX) and cloned into the pBluescript II SK vector (pBSK). The pBSKgDEGFRvIII, pBSKgDMAGE and pBSKgDGLEA plasmids were cleaved with NheI and XbaI restriction enzymes (Fermentas, Thermo Scientific, Cat. #FD0974, #FD0684), whose sites were included at the beginning and end, respectively, of all fragments. The cleaved gDEGFRvIII, gDMAGE, gDGLEA fragments were cloned into the pVAX (Invitrogen, Cat. V260-20) vector. Cloning was confirmed by restriction enzyme digestion, PCR and DNA sequencing. The pBSKgDEGFRvIII, pBSKgDMAGE and pBSKgDGLEA plasmids were also used as templates for EGFRvIII, MAGE and GLEA amplification. Reactions were performed using the following primers (IDT, Integrated DNA Technologies):

Forward

EGFRpVAX_S

5'-

ACTGACTAGCACCATGCTGGAGGAAAAGAAAGGTAATTATGTGGTGACA

GATCACG-3'

MAGEpVAX_S

5'-TACTAGCTAGCACCATGCCTCTTGAGCAGAG-3'

GLEApVAX_S

5'-TACTAGCTAGCACCATGACAAAGCATCCACC-3'

Reverse

EGFRpVAX_R 5'-ATTCTAGATCAGCAGTGGGGGCCGTC-3'

MAGEpVAX_R 5'-ATTCTAGATCACTCTTCCCCCTCTC-3'

GLEApVAX_R 5'-ATTCTAGATCACTTCTTTGGCTTCAC-3'

In the forward primer sequences, the bold letters represent the Kozak sequence, and the NheI restriction site is underlined. In the reverse primers, the bold letters represent stop codons, and the XbaI restriction site is underlined. The conditions for PCR were as follows: a 100 μ L PCR mixture composed of 100-150 ng of template, 0.2 mM each dNTP (Invitrogen, Cat. 10297-018), 0.1 mM each primer and 5 units of High Fidelity PCR enzyme mix (Fermentas, Thermo Scientific, Cat.#K0192). The following amplification program was used: 94°C for 5 min, 24 cycles of 94°C for 1 min, 48°C for 1 min, and 72°C for 1 min (GLEA, 2 min), with a final step at 72°C for 10 min. The amplified EGFRvIII, MAGE and GLEA fragments were cleaved with NheI and XbaI restriction enzymes and cloned into pVAX without gD. Cloning was confirmed by restriction enzyme digestion, **PCR and DNA sequencing**.

Large-scale purification of the DNA vaccines pVAXgDEGFRvIII, pVAXgDMAGE, pVAXgDGLEA, pVAXEGFRvIII, pVAXMAGE and

pVAXGLEA was conducted by ion-exchange chromatography using an Endofree Plasmid Giga Kit (Qiagen, Cat.12391) according to the manufacturer's instructions. DNA vaccines were quantified by spectrophotometry (Nanodrop 1000™, Thermo Scientific, Wilmington, DE 19810 USA) and analyzed for the endotoxin presence using a QCL-1000 LAL Kit (Cambrex, Cat. 50-647U) according to the manufacturer's instructions.

Construction of expression vectors

The pBSKgDEGFRvIII, pBSKgDMAGE and pBSKgDGLEA plasmids were used as templates for EGFRvIII, MAGE and GLEA amplification. Reactions were performed using the following primers:

Forward

EGFRpET28a_S 5'-
TACTAGCTAGCATGCTGGAGGAAAAGAAAG-3'

MAGEpET28a_S 5'-
TACTAGCTAGCATGCCTCTTGAGCAGAG-3'

GLEApET28a_S 5'-TACTAGCTAGCATGACAAAGCATCCACC-3'

Reverse

EGFRpET28a_R 5'-GATCTCGAGTCAGCAGTGGGGGCCGTC-3'
MAGEpET28a_R 5'-
GATCTCGAGTCACTCTTCCCCCTCTCTC-3'

GLEApET28a_R 5'-GATCTCGAGTCACTTCTTTGGCTTCAC-3'

In the forward primer sequences, the underlined letters represent the NheI restriction site. In the reverse primer sequences, the bold letters represent stop codons, and the XhoI restriction site is underlined. The conditions for PCR were as follows: a 100 µL PCR mixture composed of 100-150 ng of template, 0.2 mM each dNTP, 0.1 mM each primer and 5 units of High Fidelity PCR enzyme mix. The amplification program was the same as described above. The amplified EGFRvIII, MAGE and GLEA fragments were cleaved with NheI and XhoI (Fermentas, Thermo Scientific, Cat. #ER0695) restriction enzymes and cloned into the pET28a vector (pET) (Novagen, Cat. 69864-3). Cloning was confirmed by restriction enzyme digestion, PCR and DNA sequencing.

Expression and purification of the recombinant proteins

The EGFRvIII, MAGE and GLEA fragments were cloned into the pET, which encodes the N-terminal 6XHis-tag. The *E. coli* BL21 and Rosetta strains were transformed with the pTEGFRvIII, pETMAGE, pETGLEA plasmids. For the production of the recombinant proteins EGFRvIII, MAGE and GLEA, the transformed bacteria were grown at 37°C in LB broth (USB, Cat. 75852) supplemented with 50 µg/mL kanamycin (Gibco, Cat. 905037IP) to an optical density of 0.5-0.6 at 600 nm (OD₆₀₀=0.5-0.6), and protein expression was induced with 0.5 mM IPTG (isopropylthio-β-galactoside) (Invitrogen, Cat. 15529-019) at 30°C overnight (MAGE was induced with 0.5 mM IPTG at 37°C for approximately 4 h, and GLEA was induced with 0.2 mM IPTG at 37°C for approximately 4 h). The cell pellets were collected, analyzed by 10% SDS-PAGE [46], with Coomassie Brilliant Blue R-250 (USB, Cat. 32826) and used for protein purification.

Recombinant proteins were purified by affinity chromatography on Ni-NTA (GE Healthcare, Cat. 17-5318-02) resin using 6XHis-tag according to the manufacturer's protocol and analyzed by 10% SDS-

PAGE with Coomassie Brilliant Blue. The EGFRvIII and GLEA recombinant proteins were purified under denaturing conditions (8 M urea); MAGE, under native conditions. The purified protein fractions were dialyzed against PBS (phosphate-buffered saline), concentrated using a GE Healthcare Vivaspin (Cat. 28-9323-61) and quantified using a Coomassie Plus™ Protein assay kit (Pierce, Thermo Scientific, Cat. #1856210) [47]. When the EGFRvIII and GLEA proteins precipitated after dialysis, they were centrifuged, and the supernatants were used in the assays.

Production of antibodies against the recombinant proteins

Female BALB/c mice at 6-8 weeks of age from the Animal Breeding Center of the School of Medicine of Ribeirão Preto-University of São Paulo were used in this protocol. All procedures involving the handling and killing of animals were performed according to approved protocols and in accordance with recommendations for the proper use and care of laboratory animals at the School of Medicine of Ribeirão Preto-University of São Paulo (number 082/2009). Three mice were immunized subcutaneously in the back with 50 µg of recombinant protein (MAGE or GLEA) or precipitated protein (EGFRvIII) emulsified with Freund's incomplete adjuvant (Sigma, Cat F5506) at a ratio of 1:1 (oil:aqueous phase), which was administered 3 times in 12 day intervals. Then, the mice were sacrificed and bled by cardiac puncture 20 days after the last immunization. Sera were obtained from the whole blood samples, pooled and used in the DNA vaccine characterization.

DNA vaccine characterization

Human embryonic kidney 293 cells were cultivated in 24-well culture plates (Falcon, Cat. 353047) at a density of 2 x 10⁵ cells per well in 1 ml of DMEN medium (Gibco, Cat. 12100-046) supplemented with 10% fetal bovine serum (FBS) (Gibco, Cat. 12657-029) and 1% penicillin/streptomycin (Sigma, Cat. A5955) and incubated for 24 h at 37°C in an atmosphere of 5% CO₂. Next, the cells were transfected with 500 ng of DNA vaccine and CaCl₂ according to the protocol described by Kingston et al. and incubated again [48]. Negative control cells were transfected with the pVAX vector. After 24 h, the transfected cells were harvested, washed with PBS, lysed with RIPA buffer and evaluated by western blot. Cell samples were analyzed by 10% SDS-PAGE, transferred onto a nitrocellulose membrane (Life Technologies, Cat. 1465MB). The membrane was blocked with PBS supplemented with 0.05% Tween 20 (Vetec, Cat. 1280) (PBS-T) and 2% bovine serum albumin (InLab, Cat. 1870) for 2 h at room temperature and then reacted with anti-gD (1:20000, kindly provided by Luis Carlos de Souza Ferreira) or sera (item Production of antibodies against the recombinant proteins) containing anti-recombinant protein antibody (1:1000) (anti-EGFRvIII, MAGE or GLEA) overnight at 4°C. After the membrane was washed three times with PBS-T, it was incubated with anti-mouse IgGHRP (1:5000) (Invitrogen, Molecular Probes, Cat. F21453) for 1 h at room temperature. The immunoreactive protein bands were visualized using the peroxidase substrate 3,3'-diaminobenzidine (DAB) (Vector, Peroxidase Substrate Kit SK-4100).

Mouse immunization

Groups of 8 to 12 female C57BL/6 mice at 6-8 weeks of age from the same animal breeding center were immunized with the DNA vaccines (pVAXgDEGFRvIII, pVAXgDMAGE, pVAXgDGLEA, pVAXEGFRvIII, pVAXMAGE and pVAXGLEA) 3 times intramuscularly in 12 days intervals. The control group was

immunized with the pVAX vector. Each dose of 100 µg of DNA was diluted in 25% saccharose, divided into two 50 µl aliquots and injected into the tibialis anterior muscle of each hind limb.

Assessment of humoral and cellular immune responses

Twenty days after the last immunization, the mice were sacrificed and bled, and the spleens were removed. Sera were obtained from the whole blood samples, and cell suspensions were obtained from the macerated spleen samples. Cells were treated for 2 min with 2 ml ammonium chloride potassium buffer to lyse red blood cells, washed with PBS and suspended in RPMI 1640 medium (Sigma, Cat. R6504) supplemented with 10% FBS, 1% penicillin/streptomycin, 30 µg/ml polymyxin B (Sigma, Cat. P4932), 10 µg/ml gentamicin (Gibco, Cat. 15710-064) and 50 µM 2-mercaptoethanol (Sigma, Cat. M7522). Then, 5×10^6 cells per well were cultured in 1 ml of medium in the presence of 20 µg/ml EGFRvIII, MAGE or GLEA recombinant proteins for 48 h at 37°C in an atmosphere of 5% CO₂. Positive and negative controls were treated with 40 µg/ml concanavalin A (ConA) (Sigma, Cat. C2631) and medium only, respectively.

Sera from vaccinated mice were tested for the presence of IgG1 and IgG2a antibodies against the recombinant proteins by ELISA. Briefly, 96-microwell plates (Maxisorb, Nunc, Cat. 442404) were coated with 100 µl of coating buffer (15 mM Na₂CO₃ and 35 mM NaHCO₃, pH 9.6) containing 5 µg/ml recombinant protein and incubated overnight at 4°C. After the plates were washed with PBS-T, they were blocked with 200 µl of PBS-T containing 10% FBS (block solution) for 1 h at 37°C. After the plates were washed again, 100 µl of serum samples diluted (1:10) in blocking solution was added to the plates, incubated for 2 h at 37°C and washed. Next, 100 µl of biotinylated anti-IgG1 (A85-1) or anti-IgG2a (R19-15) (BD Bioscience) diluted 1:1000 in blocking solution was added, and the plates were incubated for 1 h at 37°C. Then, the plates were washed and incubated with 100 µl of streptavidin-biotin-horseradish peroxidase (1:1000 dilution; Vector, Vectastain ABC-kit) for 30 min at room temperature. Detection was performed with 100 µl of TMB substrate (BD Bioscience, Cat. 555214). After 30 min at room temperature, the enzyme reaction was stopped by 50 µl of 16% sulfuric acid, and the absorbance was measured at 490 and 570 nm.

Cytokine production in the supernatants of cell cultures was evaluated by ELISA. Briefly, 96-microwell plates were coated with 100 µl of coating buffer (0.1 M Na₂HPO₄, pH 9.0) containing capture antibody [IFN-γ (R4-6A2) (Cat. 551216) and IL-5 (TRFK5) (Cat. 554393) at 1 µg/ml; IL-10 (JES5-2A5) (Cat. 551215) at 2 g/ml] (BD Biosciences) and incubated overnight at 4°C. After the plates were washed with PBS-T, they were blocked with 200 µl of blocking solution for 1-2 h at room temperature. After the plates were washed again, 100 µl of supernatant samples was added to the plates, incubated overnight at 4°C and washed. Next, 100 µl of biotin-labeled secondary antibody [IFN-γ (XGM1.2) (Cat. 554410) and IL-5 (TRFK4) (Cat. 554397) at 0.5 µg/ml; IL-10 (SXC-1) (Cat. 554423) at 1 µg/ml] (BD Biosciences) diluted in blocking solution was added, and the plates were incubated for 1 h at room temperature. Then, the plates were washed and incubated with 100 µl dilution of streptavidin-biotin-horseradish peroxidase (IFN-γ, 1:500; IL-10 and IL-5, 1:1000) for 30 min at room temperature. Detection was performed with 100 µl of TMB substrate. After incubation for 30 min at room temperature, the enzyme reaction was stopped by 50 µl of 16% sulfuric acid, and the absorbance was measured at 490 and 570 nm.

Statistical analysis

Comparisons were performed using ANOVA with Tukey's test a posteriori and GraphPad Prism 4.02 version software (GraphPad Software, San Diego, CA, USA). Statistical significance was attributed to p values <0.05.

Results

Construction of DNA vaccines

The coding sequences of EGFRvIII, MAGE-3 (MAGE) and GLEA-2 (GLEA) proteins without their stop codons were fused to the gD nucleotide sequence in silico. The fusion to gD resulted in the deletion of the gD central portion with 214 amino acids. Because the EGFRvIII sequence is extremely large, only the initial 900 base pairs (bp) were utilized in this assembly. gDEGFRvIII, gDMAGE, gDGLEA fragments were constructed by Epoch Biolabs and cloned into the pBluescript II SK vector (pBSK). The pBSKgDEGFRvIII, pBSKgDMAGE and pBSKgDGLEA plasmids were cleaved to remove the gD-antigen fragments and were used as templates for EGFRvIII, MAGE and GLEA amplification. All fragments were cloned into the pVAX1 (pVAX) vector. Because pVAX is a transcription plasmid, all the fragments already had the Kozak sequence (ACCATG) [49]. Restriction analyses (Figure 1) and sequencing (data not shown) confirmed that the DNA vaccines were correctly constructed. As shown in Figure 1, pVAXgDEGFRvIII, pVAXgDMAGE, pVAXgDGLEA, pVAXEGFRvIII, pVAXMAGE and pVAXGLEA constructs presented sizes that matched the vector associated with the insert: 4,357 bp, 4,399 bp, 5,086 bp, 3,820 bp, 3,859 bp and 4,546 bp, respectively.

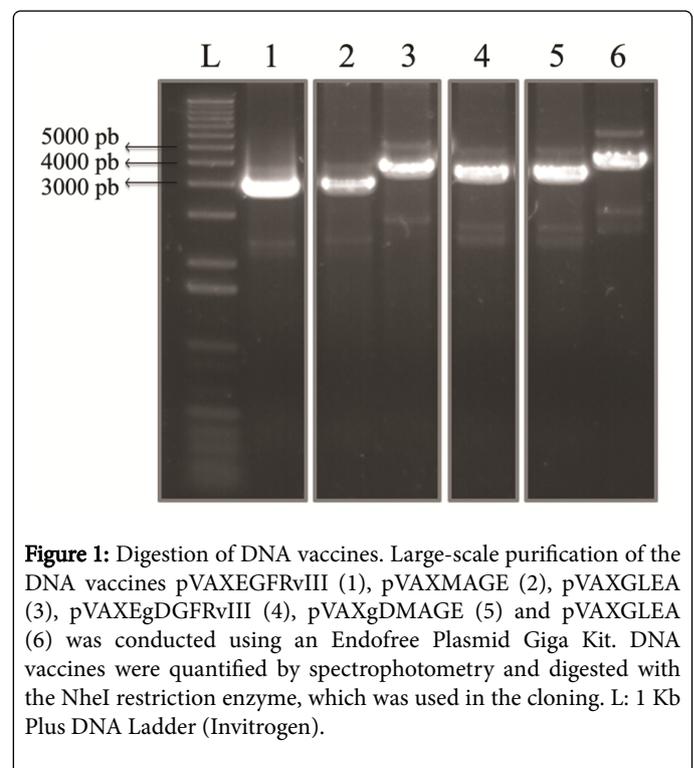
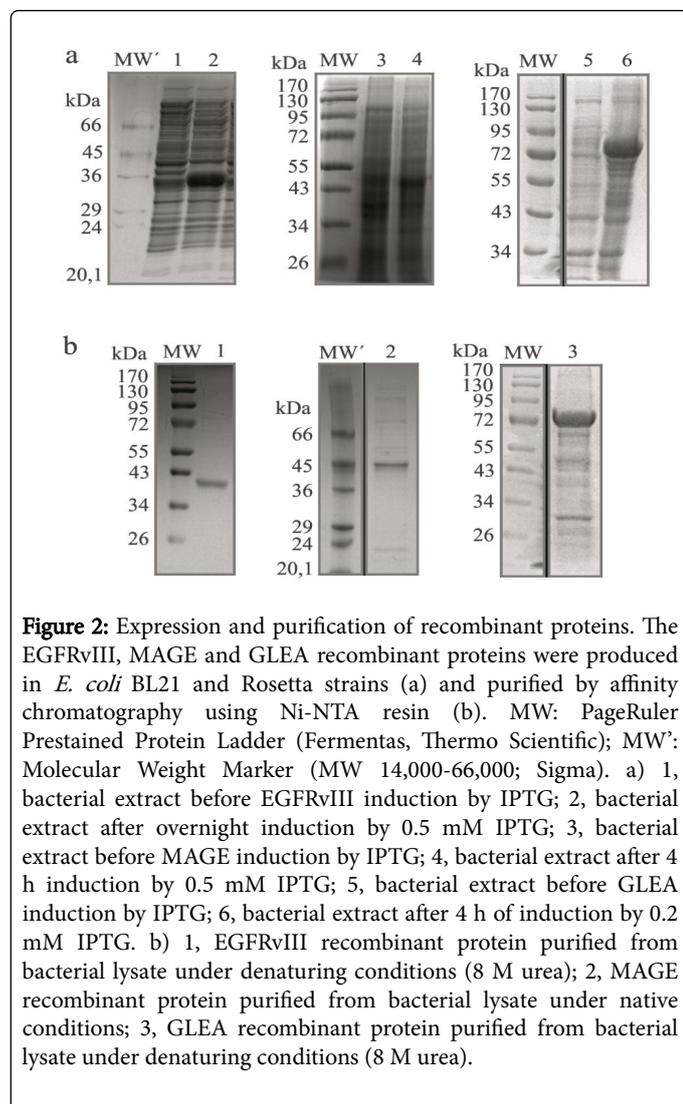


Figure 1: Digestion of DNA vaccines. Large-scale purification of the DNA vaccines pVAXEGFRvIII (1), pVAXMAGE (2), pVAXGLEA (3), pVAXEgDGFRvIII (4), pVAXgDMAGE (5) and pVAXGLEA (6) was conducted using an Endofree Plasmid Giga Kit. DNA vaccines were quantified by spectrophotometry and digested with the NheI restriction enzyme, which was used in the cloning. L: 1 Kb Plus DNA Ladder (Invitrogen).

Expression and purification of the recombinant proteins

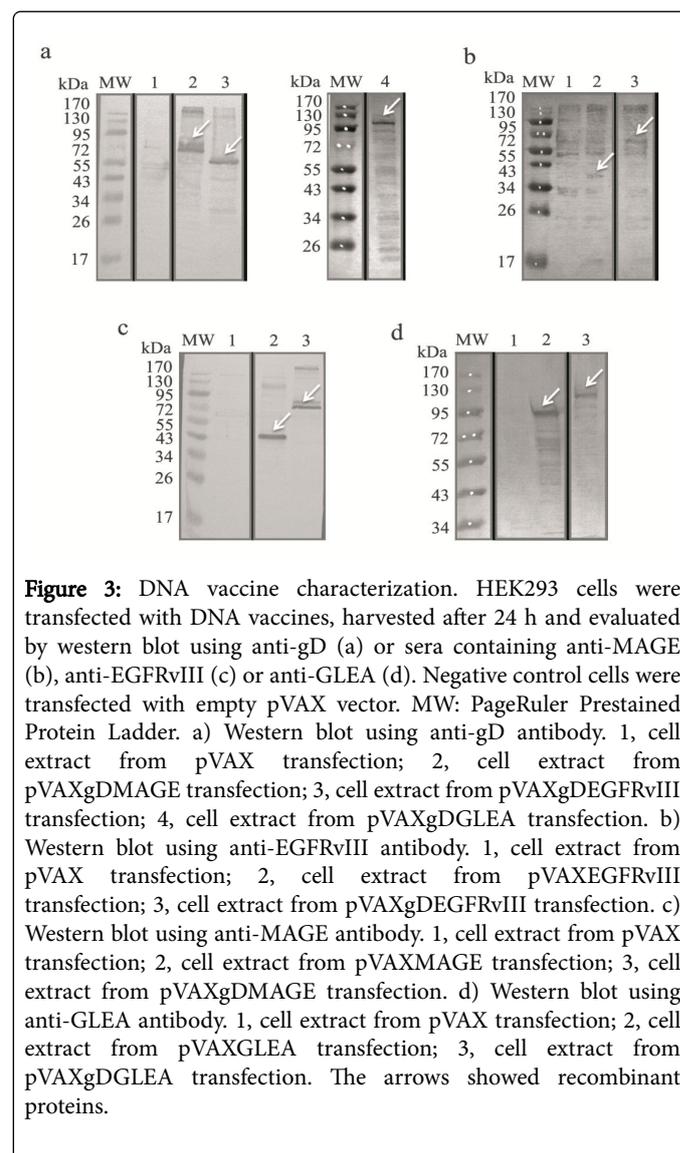
EGFRvIII, MAGE and GLEA were also amplified from pBSKgDEGFRvIII, pBSKgDMAGE and pBSKgDGLEA plasmids for cloning into the *E. coli* expression vector pET. The recombinant proteins were expressed and analyzed by 10% SDS-PAGE (Figure 2a). The recombinant EGFRvIII, MAGE and GLEA proteins were separated by SDS-PAGE and were 36 kDa, 43 kDa and 72 kDa, respectively. The MAGE and GLEA proteins showed larger molecular masses than expected (37 and 64 kDa, respectively). The recombinant EGFRvIII, MAGE and GLEA proteins were purified by affinity chromatography and analyzed by 10% SDS-PAGE (Figure 2b). The recombinant proteins were used for mouse immunization, antibody production and immunogenicity assays.



DNA vaccines characterization

To verify whether the DNA vaccines could express the expected proteins, human embryonic kidney 293 (HEK293) cells were transiently transfected with the constructs. One day later, cell extracts were obtained, and the proteins were detected by western blot with the appropriate antibodies (Figure 3). The extract from cells transfected with the empty pVAX vector failed to bind to gD, EGFRvIII, MAGE

and GLEA-specific antibodies. Anti-gD antibody stained extracts from cells transfected with vaccines expressing the gDEGFRvIII, gDMAGE and gDGLEA fusion proteins (Figure 3a). Cells transfected with pVAXgDEGFRvIII and pVAXEGFRvIII were stained by anti-EGFRvIII antibody specific to the corresponding tumor antigen (Figure 3b). Extracts from cells transfected with pVAXgDMAGE and pVAXMAGE were stained by anti-MAGE antibody (Figure 3c), and extracts from cells transfected with pVAXgDGLEA and pVAXGLEA were stained by anti-GLEA antibody (Figure 3d). Collectively, these results demonstrate that the proteins encoded by the DNA vaccines were correctly expressed by the cellular machinery.



Assessment of humoral and cellular immune responses

C57BL/6 mice received three doses of the DNA vaccines (pVAXgDEGFRvIII, pVAXgDMAGE, pVAXgDGLEA, pVAXEGFRvIII, pVAXMAGE or pVAXGLEA) at a 12 days interval regimen. Twenty days after the last immunization, blood and spleen samples were collected. Sera were obtained from the whole blood samples and were tested for the presence of IgG1 and IgG2a antibodies against the recombinant proteins EGFRvIII, MAGE and GLEA by

ELISA. The production of specific IgG2a antibodies was inconsistent for the pVAXgDEGFRvIII (Figure 4a) and pVAXgDMAGE (Figure 4b) groups. Only the pVAXgDGLEA group produced a high level of

specific IgG2a antibodies (Figure 4c). Specific IgG1 antibodies were not detected in any group (data not shown).

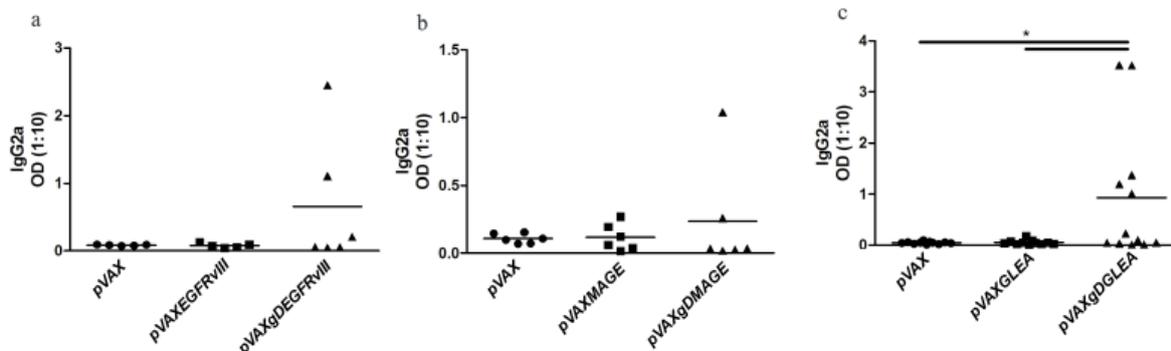


Figure 4: Humoral immune response induced by DNA vaccines. Sera from vaccinated mice with three doses of 100 μ g of DNA vaccines were collected 20 days after the last immunization and tested for the presence of IgG2a antibodies against recombinant proteins by ELISA. a) Anti-EGFRvIII detection. The data presented are from one experiment (6 mice per group). b) Anti-MAGE detection. Representative data presented are from one experiment (6 mice per group). c) Anti-GLEA detection. The data presented are from two experiments (12 mice per group). * $p < 0.05$, one-way ANOVA and Tukey's posttest.

To characterize antigen-specific cell-mediated responses, cells were obtained from macerated spleen samples and were stimulated *ex vivo* with the recombinant proteins or with concanavalin A (ConA) as a positive control. The negative control was performed with unstimulated cells. Cytokine production by the stimulated cells was quantified by ELISA using culture supernatants. IFN- γ (Figure 5) and

IL-10 (Figure 6) production was significantly higher in the pVAXgDEGFRvIII (Figure 5a and 6a), pVAXgDMAGE (Figure 5b and 6b) and pVAXgDGLEA (Figure 5c and 6c) groups compared with the other groups. IL-5 production was not detected in any group (data not shown).

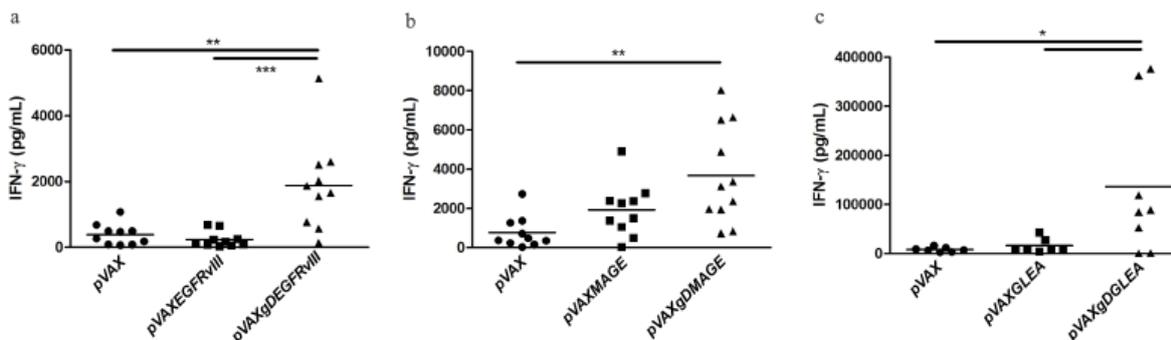


Figure 5: Assessment of cellular immune responses induced by DNA vaccines by measuring IFN- γ production. Mice were vaccinated with three doses of 100 μ g of DNA vaccines. Spleen cells were collected 20 days after the last immunization and stimulated *ex vivo* for 48 h. IFN- γ production levels in the culture supernatants were quantified by ELISA. a) Culture stimulated with EGFRvIII protein. The data presented are from two experiments (12 mice per group). b) Culture stimulated with MAGE protein. The data presented are from two experiments (12 mice per group). c) Culture stimulated with GLEA protein. The data presented are from one experiment (8 mice per group). The differences in the cellular responses were determined by one-way ANOVA and Tukey's posttest. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. The negative control was performed without any stimulus, and if detected, cytokine production was discounted for data tabulation of other stimuli. The positive control was performed using ConA as the polyclonal stimulus.

Discussion

Despite multimodal treatment with maximal surgical resection followed by temozolomide and radiation, GBM remains a recurrent and fatal cancer [4,5]. Thus, immunotherapy combined with conventional therapy constitutes an alternative intervention, providing the advantage of inducing a long-term, specific immune response that

could prevent GBM recurrence. An effective vaccine for cancer should elicit not only a humoral response but also a cellular response. However, vaccines containing antigens alone do not induce efficient cellular responses. Thus, antigens need to be combined with an efficient adjuvant or delivery system that can be used in humans. Therefore, we developed DNA vaccines encoding GBM antigens fused

to gD from HSV-1. Our results demonstrated that our DNA vaccines were constructed correctly and that the proteins were expressed in transfected HEK293 cells.

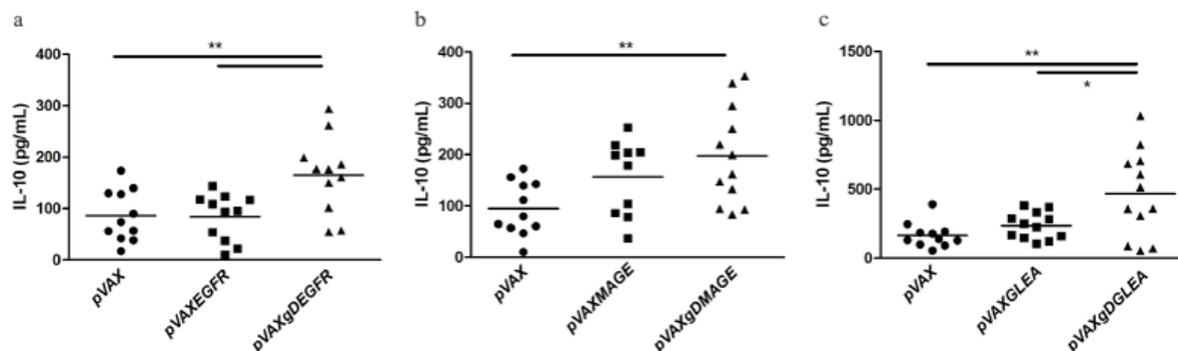


Figure 6: Assessment of cellular immune responses induced by DNA vaccines by measuring IL-10 production. Mice were vaccinated with three doses of 100 µg of DNA vaccines. Spleen cells were collected 20 days after the last immunization and stimulated ex vivo for 48 h. IL-10 production levels in the culture supernatants were quantified by ELISA. a) Culture stimulated with EGFRvIII protein. The data presented are from two experiments (12 mice per group). b) Culture stimulated with MAGE protein. The data presented are from two experiments (12 mice per group). c) Culture stimulated with GLEA protein. The data presented are from two experiments (12 mice per group). The differences in the cellular responses were determined by one-way ANOVA and Tukey's posttest. * $p < 0.05$, ** $p < 0.01$. The negative control was performed without any stimulus, and if detected, cytokine production was discounted for data tabulation of other stimuli. The positive control was performed using ConA as the polyclonal stimulus.

Previous observations have indicated that gD can enhance the immunogenicity of antigens present in DNA vaccines when these antigens are fused to gD [32-34,50]. Antigens and secondary signals are required for the activation of adaptive immune responses. Secondary signals are delivered by co-stimulatory molecules, and gD can perform this function when interacting with cell surface receptors, thus activating type I IFN and IL-12 production by DCs [43,44]. Lasaro et al. also suggested that a secondary signal could originate from the inhibition of the HVEM-BTLA/CD160 inhibitory pathway by gD, which interacts with HVEM in the same site to which other molecules bind [32,51]. BTLA is an inhibitory receptor present in T cells that inhibits IL-2 secretion when activated. BTLA-deficient T cells display increased proliferation in specific T lymphocytes stimulated by antigen-DCs [52]. CD160 presents BTLA-like features, thus inhibiting T CD4+ activation [42]. Therefore, blocking the HVEM-BTLA/CD160 interaction can enhance the T cell response.

In this study, DNA vaccines codifying only tumor antigens showed low immunogenicity. Mice immunized with pVAXEGFRvIII, pVAXMAGE and pVAXGLEA did not present relevant secondary humoral or cellular immune responses. Many studies have shown that DNA vaccines can stimulate both humoral and cellular immune responses [53-55]. However, most of the proteins produced by DNA immunization are not able of priming the immune response by themselves, thus, is usually required to do the enhancement of the potency of genetic vaccination [56-58]. Satisfying this condition, the pVAXgDEGFRvIII, pVAXgDMAGE and pVAXgDGLEA vaccines encoding the tumor antigens genetically fused to the gD protein were able to induce cellular immune responses, despite low induced humoral responses. The immune effects induced by these vaccines are suggestive that the structural integrity of the gD-HVEM binding domains is preserved. The induction of adaptive immune response (humoral and cellular) was evaluated after stimulation with the recombinant proteins EGFRvIII, MAGE and GLEA, which were

expressed in *E. coli* and purified by affinity chromatography. The MAGE and GLEA recombinant proteins showed larger molecular masses than expected, as demonstrated previously by Kocher et al. [59]. The authors showed that the MAGE protein, whether recombinant or native in melanoma cells, presented larger molecular masses than expected [59]. Because the MAGE protein does not show post-translational modifications, the authors suggested that the size could be changed due to its amino acid composition [59]. The prevalence of amino acids rich in acidic residues can alter the electrophoresis pattern of the protein. The amino acid composition may also explain the increased size of the GLEA protein, which also does not undergo post-translational modifications when expressed in *E. coli*.

Analysis of the cellular immune responses showed that the IFN- γ production in the supernatants of spleen cells from mice vaccinated with pVAXgDEGFRvIII, pVAXgDMAGE and pVAXgDGLEA were significantly higher than those levels verified in the animals of other immunization groups. This result indicates that tumor antigens associated with gD drive the T cell response toward a Th1-type immune response profile. Immunomodulatory effects of gD were also demonstrated in other studies with DNA vaccines codifying E5, E6 and E7 tumor antigens fused to gD [32-34]. Mice immunized with these DNA vaccines presented marked increases in specific CD8+ T cells [32-34]. In another study by the same group, trivalent DNA vaccines that simultaneously encoded HIV (p24), HSV (gD) and HPV (E7) antigens stimulated functional and protective p24-, gD- and E7-specific CD8+ T cell responses in vaccinated mice, thus promoting efficient anti-virus (HIV/HSV-1) and anti-tumor (HPV) effects [60].

For all vaccination strategies, cell-mediated immune response and IFN- γ production have been considered essential for the generation of a long-lasting specific immune response against tumor cells. IFN- γ participates in immunosurveillance that protects against

spontaneously arising, transplantable, chemically induced tumors [61]. The effects of IFN- γ on host cells include activation of the Th1-cell lineage, enabling macrophage activation and CTL maturation [62,63], and inhibition of regulatory T cell generation and/or activation [64]. In contrast, IFN- γ also presents a direct anti-tumor mechanism that promotes apoptosis through its effects on the expression of caspases, FAS and TRAIL [65-67], thus inhibiting cellular proliferation and angiogenesis [68,69].

Spleen cells from mice vaccinated with pVAXgDEGFRvIII, pVAXgDMAGE and pVAXgDGLA stimulated with recombinant proteins produced significantly higher levels of IL-10 than mice immunized with the other constructs. The functions of some cytokines produced in the tumor environment may have different effects when compared with situations in which the same cytokine is produced outside the tumor environment [70]. This situation has been confirmed with IL-10; its role in tumor immunology is contradictory. IL-10 was initially classified as a Th2-derived cytokine [71]; however, it is produced by a range of CD4+ T cell subsets and by macrophages, DCs, B cells, eosinophils and mast cells [72]. Generally, IL-10 is considered an immunosuppressive molecule [72,73], and in glioma cancer, it is produced by cancer cells to escape the immune response [74].

However, some studies have shown the importance of IL-10 in controlling tumor cells. T cells primed by autologous DCs loaded with GBM tumor cell lysates acquired a Th1 profile and the capacity to produce IL-10 [75]. This production correlated with antitumor T cell activity [75]. In a vaccine strategy to protect against tumor challenge, Shin and collaborators showed that IL-10 administration promotes the proliferation and maintenance of T CD8+ -activated antigen-specific cells [76]. These cells produced IFN- γ and displayed an increased cytotoxic capacity, which supports tumor reduction [76]. Other researchers demonstrated that IL-10-deficient mice developed a greater number of skin tumors and less granzymes than wild-type animals. Furthermore, the administration of IL-10 promoted tumor rejection in several tumor types even when the tumor was well established. These authors clearly showed that the anti-tumor activity of IL-10 is dependent on IFN- γ induction in CD8+ T cells. The anti-tumor CD8+ T cells present higher IL-10 receptor expression, which explains its activation via this cytokine [77]. These results indicate that IL-10 may be a helpful cytokine in immunization strategies because it improves the effectiveness of anti-tumor CD8+ T cells.

Other studies have revealed that IL-10 $^{-/-}$ mice develop many colon polyps with accelerated growth and lung metastasis when compared to control animals [78]. These animals, despite their IL-10 deficiency, have a greater number of regulatory T cells (Tregs) and myeloid-derived suppressor cells (MDSCs), which increase immunosuppression and inhibit the anti-tumor immune response [78]. Instead, the myeloid cells of these animals expressed higher levels of IL-1 α and IL-1 β , which are pro-angiogenic and pro-tumorigenic [78]. The inhibitory signaling of these cytokines decreases angiogenesis and Tregs and increases CD8+IFN- γ + T cells resulting in tumor reduction [78]. Thus, tumor development in IL-10 $^{-/-}$ animals results from a linked mechanism of immunosuppression and inflammation that impairs anti-tumor responses.

In contrast, a new theory suggests that Th1 cells are regulated via the co-induction of IL-10 in addition to IFN- γ in the same cells [79,80]. These cells that produce IL-10 develop from IFN- γ + Th1 cells when appropriate signals are received and represent the 'endpoint' of an effective immune response (one component of the life cycle of a Th1 cells) [81]. Jankovic and collaborators suggested an explanation to this

theory, namely, that IFN- γ is important cytokine for combatting pathogens and that IL-10 protects against tissue injury that can be triggered by immune responses [82]. Thus, Th1 IFN- γ +IL-10+ cells promote and control immune responses by effector and regulatory cytokine induction, following recognition of the same antigen [81]. The two roles of IL-10, i.e., favoring or controlling the immune response, are important for destroying tumors and for controlling immune responses that should not cause damage to normal tissues.

In summary, this study indicates the potential value of DNA vaccination using the expression of GBM antigens genetically fused with the HSV-1 gD. DNA vaccination with chimeric tumor antigens stimulated cell-mediated immunity, revealed by the specific IFN- γ and IL-10 responses, which is considered essential for combating tumors. Collectively, these results provide evidences for the potential application of an active immunotherapy method to be used with conventional therapy for GBM treatment.

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Conflicts of Interest

No other funding or conflicts of interest to declare.

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