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Purified Herpes Simplex Type 1 Glycoprotein D (gD) Genetically Fused with the Type 16 Human Papillomavirus E7 Oncoprotein Enhances Antigen-Specific CD8$^+$ T Cell Responses and Confers Protective Antitumor Immunity

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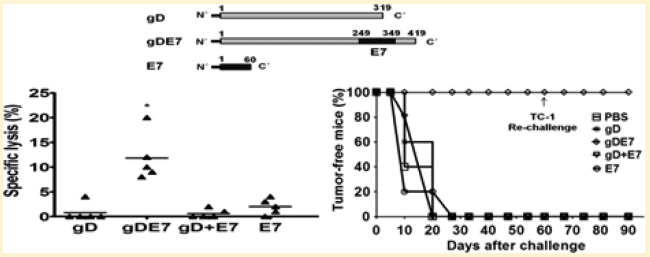
ABSTRACT: Type 1 herpes virus (HSV-1) glycoprotein D (gD) enhances antigen-specific immune responses, particularly CD8$^+$ T cell responses, in mice immunized with DNA vaccines encoding hybrid proteins genetically fused with the target antigen at a site near the C-terminal end. These effects are attributed to the interaction of gD with the herpes virus entry mediator (HVEM) and the concomitant blockade of a coinhibitory mechanism mediated by the B- and T-lymphocyte attenuator (BTLA). However, questions concerning the requirement for endogenous synthesis of the antigen or the adjuvant/antigen fusion itself have not been addressed so far. In the present study, we investigated these points using purified recombinant gDs, genetically fused or not with type 16 papilloma virus (HPV-16) E7 oncoprotein. Soluble recombinant gDs, but not denatured forms, retained the ability to bind surface-exposed cellular receptors of HVEM-expressing U937 cells. In addition, *in vivo* administration of the recombinant proteins, particularly gD genetically fused with E7 (gDE7), promoted the activation of dendritic cells (DC) and antigen-specific cytotoxic CD8$^+$ T cells. More relevantly, mice immunized with the gDE7 protein developed complete preventive and partial therapeutic antitumor protection, as measured in mice following the implantation of TC-1 cells expressing HPV-16 oncoproteins. Collectively, these results demonstrate that the T cell adjuvant effects of the HSV-1 gD protein did not require endogenous synthesis and could be demonstrated in mice immunized with purified recombinant proteins.



KEYWORDS: HPV-16, papillomavirus vaccines, gD, HSV-1, E7 oncoprotein, anticancer vaccines

1. INTRODUCTION

Modulation of T cell activity is a challenge for modern vaccinology, and the search for new adjuvants that can induce appropriate activation of T cells, particularly antigen-specific cytotoxic CD8$^+$ T cells, is a top priority.[1,2] Recent results demonstrated that modulation of herpes virus entry mediator (HVEM), a member of the tumor necrosis factor receptor (TNFR) family, can exert strong immunomodulatory effects that influence the survival, proliferation, and differentiation of T cells.[3] HVEM, as well as its agonists, is broadly expressed on a variety of immune system cells, including hematopoietic cells such as resting T cells, monocytes and immature dendritic cells (DC).[4−8] When bound to LIGHT or lymphotoxin α (LTα), which are members of the TNF superfamily, HVEM deploys costimulatory signals that ultimately lead to T cell activation following an encounter with an activated APC.[9,10] However, when bound to B and T lymphocyte attenuator (BTLA) or CD160, which are members of the immunoglobulin superfamily, HVEM triggers coinhibitory signals that dampen T cell activation, leading to an anergic state.[7,11]

In addition to binding to endogenous ligands, HVEM acts as a virus receptor and is recognized by the herpes simplex virus (HSV) glycoprotein D (gD).[12] HVEM binding to gD competitively excludes binding of the coinhibitory factors but does not interfere with the costimulatory agonists. Therefore, in addition to mediating virus entry into different immune system cells, gD binding alters the complex signaling regulatory pathways mediated by HVEM during infection with HSV-1 or HSV-2.[13] The HSV-1 gD protein is an outer-face envelope protein consisting of 394 amino acids with a signal peptide of 20 amino acids; following processing, HSV-1 gD yields a mature protein of 374 amino acids. Most of the protein is located on the outer side of the membrane (amino acids 1 to 319), with a short membrane anchor sequence (amino acids 320 to 344) followed by a C-terminal cytoplasmic tail (amino acids 344 to 374).[14] Structural analyses and experiments using monoclonal antibodies with known binding sites demonstrated that gD binding to HVEM is mediated by two N-terminal stretches defined by amino acid residues 7 to 15 and 24 to 32, which are located in a

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conformational flexible domain that folds back to the surface-exposed distal portion of the protein.[15−17] Indeed, *in vitro* studies showed that the interaction of gD with the HVEM receptor are drastically increased after the deletion of a C-terminal portion of the protein, suggesting that, under native conditions, HVEM binding requires complex conformational changes that involve the C-terminal portion of the protein.[18]

Previous reports based on experimental immunizations with DNA vaccines or recombinant adenovirus vectors showed that expression of the HSV-1 gD protein genetically fused with type 16 human papilloma virus (HPV-16) oncoproteins at a specific site near the gD C-terminal end induces enhanced and long-lived antigen-specific $CD8^+$ T cell responses.[19−21] These responses could not be achieved with vaccines encoding only the HPV-16 oncoproteins. Indeed, the enhanced $CD8^+$ T cell responses induced by the E7 protein correlated with preventive and therapeutic antitumor effects observed in mice immunized with DNA vaccines encoding hybrid gD proteins.[19,21] The search for therapeutic vaccines specifically targeting HPV-induced tumors has been significantly boosted by the availability of a murine challenge model based on TC-1 cells that constitutively express the HPV-16 E7 and E6 oncoproteins.[22,23] Based on this experimental model, candidate vaccines that show anticancer effects can easily be screened and improved before selection for clinical trials.[24]

In the present study, we set up conditions to evaluate the immunogenicity and adjuvant effects of purified recombinant gD protein that has been engineered to delete part of the C-terminal end and, thus, favor production of a soluble protein in a prokaryotic expression system. The recombinant gD forms, either admixed or genetically fused with the HPV-16 E7 oncoprotein, were tested for adjuvant effects on the induced antibody and T cell-mediated responses in vaccinated mice. The induced E7-specific immune responses as well as the observed antitumor effects demonstrate that purified gDE7 exerts $CD8^+$ T cell adjuvant effects and offers encouraging prospects for the development of new tools for modulating T cell responses.

2. MATERIAL AND METHODS

2.1. Mice, Cell Lines and Antibodies. Male C57BL/6 mice (6−8 weeks old) were provided by the animal facility unit of the Department of Parasitology at the University of São Paulo. All animal handling and immunization procedures were approved by the ethics committee for animal experimentation and followed standard rules approved by the Brazilian College of Animal Experimentation (COBEA). The TC-1 tumor cell line, derived from C57BL/6 lung epithelial cells transformed with the v-Ha-ras oncogene and HPV-16 E6 and E7, was provided by Dr. T. C. Wu at Johns Hopkins University, Baltimore, MD. The TC-1 cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 50 units/mL penicillin/streptomycin and kept at 37 °C and 5% CO_2. For the tumor challenge experiments, TC-1 cells were harvested by trypsinization, washed twice, and suspended in serum-free media at appropriate concentrations for injection. The U937 cell line was kindly provided by Dr. A. Condino Neto from the Department of Immunology at the University of São Paulo, São Paulo, Brazil. The U937 cells were propagated in RPMI 1640 medium supplemented with 10% FBS and 50 units/mL of penicillin/streptomycin and kept at 37 °C and 5% CO_2. Anti-gD monoclonal antibody (mAb) DL-6 and anti-HVEM mAbs CW1, CW3 and CW7 were kindly provided by Dr. Gary H. Cohen and Dr. Roselyn J. Eisenberg at the University of Pennsylvania, Philadelphia, PA. The anti-E7 monoclonal antibody was purchased at Calbiochem, and fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG was purchased at BD Biosciences.

2.2. Cloning of the gD, gDE7 and E7 Coding Sequences. The pRE4 and pRE4E7 plasmids, carrying HSV-1 gD or gD genetically fused with the HPV-16 E7 oncoprotein-encoding genes, respectively, were used as templates for PCR reactions.[19] The upstream Fw*Bam*HIgD primer (5′ TCG TCA TAG TGG GAT CCC ATG GGG GT 3′, *Bam*HI restriction site underlined) bound immediately after the sequence corresponding to the gD signal sequence and the downstream Rv*Xho*IgD primer (5′ TCA GCT CGA GGT TGT TCG GGG TG 3′, *Xho*I restriction site underlined) just before the membrane anchoring domain. The resulting DNA fragments encompass the gD-coding sequence without the signal sequence and the transmembrane anchor sequence (corresponding to amino acids 1 to 319 of the mature gD protein). The fragment amplified from pRE4E7 encompasses the gD coding sequence plus the HPV-16 E7-coding sequence cloned into the unique *Apa*I restriction site of gD (100 amino acids fused immediately after the 249th amino acid of the gD sequence of the hybrid protein). The encoded hybrid gDE7 protein contains a total of 419 amino acids. The amplification reactions resulted in two fragments with 957 bp or 1,257 bp, corresponding to the gD or gDE7 encoding genes, respectively, each of which were flanked by *Bam*HI and *Xho*I restriction sites. The amplified DNA fragments were purified with the Ilustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare Life Sciences), digested with *Bam*HI and *Xho*I, and cloned into the pET28a(+) expression vector (Novagen, Darmstadt, Germany), which was previously treated with *Bam*HI and *Xho*I restriction enzymes. The recombinant plasmids were named pETgD and pETgDE7. The plasmid inserts were sequenced and matched the gD sequence data available at the NCBI bank (accession no. L09242). The recombinant plasmids encoding the correct inserts were subsequently transformed into chemically competent *Escherichia coli* BL21 (DE3) cells. The gene encoding a truncated E7 protein, encompassing the first 60 N-terminal amino acids of the native protein, was amplified and cloned into the *Bam*HI and *Xho*I restriction sites of the pET28a-(+) vector using primers Fw*Bam*HIE7_2 (5′ GGA TCC CAT GGA GAT ACA CCT AC 3′) and Rv*Xho*IE7_2 (5′ CTC GAG TTA AAA GGT TAC AAT ATT G 3′) (restriction sites underlined). The encoded protein carries the H-$2K^b$-restricted $CD8^+$ T cell-specific epitope $RAHYNIVTF_{49-57}$ as well as both T helper- and B cell-specific epitopes of the HPV-16 E7 protein.[25] In contrast to the complete E7 protein, expression of the truncated E7 protein resulted in a partially soluble protein that could be purified from recombinant *E. coli* cells.

2.3. Purification of the Recombinant gD, gDE7 and E7 Proteins Expressed in *E. coli*. The *E. coli* BL21gD, BL21gDE7 and BL21E7 strains were cultivated overnight in LB medium at 37 °C in an orbital shaker set at 200 rpm. After inoculation of 1 L of fresh LB medium and incubation for approximately 3 h (0.5 OD_{600}), IPTG was added at a final concentration of 0.1 mM, and the cells were incubated for an additional 3 h with aeration. The cells were harvested and suspended in buffer A (100 mM Tris-HCl, 500 mM NaCl, pH 9 for the gD protein or 100 mM Tris-HCl, 500 mM NaCl, pH 7.5 for the gDE7 and E7 proteins) and lysed by mechanical shearing using an APLAB-10 homogenizer (ARTEPEÇAS, Brazil). For the purification of gD and gDE7,

differential centrifugation was performed and the inclusion bodies were collected, suspended in 10 mL of buffer A_1 (8 M urea, 100 mM Tris-HCl, 500 mM NaCl, pH 9 or 8 M urea, 100 mM Tris-HCl, 500 mM NaCl, pH 7.5 for the gD and gDE7 proteins, respectively) and gently shaken overnight at 4 °C. The solutions were centrifuged and the supernatant was filtered with a Sartorius Stedim apparatus with cellulose acetate 0.22 μm mesh filters (Biotech). Filtrates containing the recombinant proteins were purified by nickel affinity chromatography using a Histrap FF column (GE Healthcare Life Sciences) previously equilibrated with buffer A_1 in an FPLC device (Akta model, Amershan Pharmacia Biotech). The column was washed using a linear gradient of buffer A_1 to B (8 M urea, 100 mM Tris-HCl, 500 mM NaCl and 1 M imidazole, pH 9 or 8 M urea, 100 mM Tris-HCl, 500 mM NaCl and 1 M imidazole pH, 7.5 for gD and gDE7 proteins, respectively). The gD protein was eluted at imidazole concentrations ranging from 200 mM and 550 mM, and the gDE7 protein was eluted with imidazole concentrations ranging between 200 mM and 700 mM. The eluted fractions were collected, pooled and refolded using a procedure in which the denaturing agent was gradually removed following dialysis with buffers containing decreasing urea concentrations (4 M, 2 M, 1 M and 0 M). The refolding process was carried out in a cross-flow filtration device (Millipore LabScale TFF System) with 30K Pellicon membranes (Millipore). For the purification of E7, the supernatant was collected after differential centrifugation, filtered with cellulose acetate 0.22 μm mesh filters and purified by nickel affinity chromatography using the Histrap FF column previously washed with buffer A in an FPLC device. Bound proteins were eluted from the column using a linear gradient of buffer A to B (100 mM Tris-HCl, 500 mM NaCl 1 M imidazole, pH 7.5), and E7 protein was eluted with 100 mM imidazole. The final protein yield was determined with a GeneQuant spectrophotometer (GE Amersham Biosciences) and visualized on polyacrylamide gels concomitantly loaded with known BSA (bovine serum albumin) concentrations. Endotoxin levels of the protein preparations were determined using the Chromogenic Limulus Amebocyte Lysate assay (Cambrex Bio Science). The endotoxin concentrations in the preparations were equal or below 1.0 EU/μg of protein.

2.4. Immunoblot and ELISA Analyses. Protein extracts from the BL21gD, BL21gDE7 and BL21E7 strains were separated on polyacrylamide gels by SDS−PAGE electrophoresis and transferred to nitrocellulose membranes (Amersham Biosciences) in transfer buffer (39 mM glycine, 20% methanol, 0.0375% SDS, 48 mM Tris-HCl pH 7.8) at 30 mAmp. The nonspecific membrane binding sites were blocked with PBS-Tween 0.05% buffer (PBS-T) plus 1% BSA. After blocking, membranes were washed and incubated with either anti-gD (DL-6) or anti-E7 for 90 min. The membrane was washed again and incubated with peroxidase-conjugated rabbit anti-mouse IgG. Reactive bands were visualized with a chemiluminescence kit (Super Signal, Pierce), as specified by the manufacturer, and exposed to Kodak X-Omat film for 1−5 min. ELISA assays were performed with 250 ng of solid phase-bound purified gD or E7 using Maxisorp or Polysorb (Nunc) microtiter plates, respectively. After overnight incubation at 4 °C, plates were blocked with PBS-T plus 1% BSA. Serial dilutions of serum samples collected one week after each immunization dose were incubated at 37 °C for 90 min, and then incubated with peroxidase-conjugated rabbit anti-mouse IgG. The optical densities were measured at 492 nm using a MultiScan EX ELISA reader (Labsystems). The individual titers were considered to be the highest dilution of serum that gave an OD_{492} higher than 0.1. The results are presented as the mean of log antibody titers ± SD with five animals per group.

2.5. gD Cell-Binding Assays. The gD cell-binding assays were performed using monocyte-derived U937 cells known to express HVEM.[26,27] Approximately 10^6 cells were propagated in RPMI 1640 medium supplemented with 10% fetal bovine serum at 37 °C and 5% CO_2. Cells were incubated with 2 μg of either gD or gDE7 protein for one hour in serum-free medium at 37 °C. Cells were washed with medium to remove unbound proteins and incubated with an anti-gD monoclonal antibody (DL-6) for 30 min. Cells were washed to remove unbound antibody and incubated with FITC-conjugated goat anti-mouse IgG for 30 min. The labeled cells were analyzed by flow cytometry.

2.6. Immunization Regimens and TC-1 Tumor Challenge. Mice were subcutaneously (sc) immunized in the flank with 4 doses of the recombinant proteins administered at weekly intervals. The recombinant gD, gDE7 and E7 proteins were suspended in PBS in a final volume of 100 μL. The experimental groups were immunized with (i) PBS; (ii) purified gD (10 μg/dose); (iii) purified gDE7 (30 μg/dose); (iv) purified gD + purified E7 (10 μg of gD + 10 μg of E7); or (v) purified E7 (10 μg/dose). The higher concentration of inoculated gDE7 with regard to gD was established in order to keep the same amount of E7 administered to the immunized mice. Mice immunized with purified gD and E7 received the two proteins as a mixture which was inoculated in the same injection site. To determine whether preventive antitumor protection could be induced, mice were inoculated with 7.5×10^4 TC-1 cells/mouse by subcutaneous injection in the right flank two weeks after the last immunization. The development of therapeutic antitumor protection was determined by inoculating with the same number of TC-1 cells one day before the administration of the first vaccine dose. Groups of five mice were used to evaluate the antitumor protection effect and the induction of cellular immune responses. Tumor growth was monitored by visual inspection and palpation once a week through at least 60 days, and cellular immune responses were analyzed two weeks after TC-1 challenge.

2.7. Determination of the $CD8^+$/IFN-γ^+ T Cell Response by Intracellular Cytokine Staining. Intracellular IFN-γ staining of peripheral blood mononuclear cells (PBMC) was performed two weeks after challenge with TC-1 cells. Blood cells were harvested and treated with ACK lysis buffer for 5 min on ice to eliminate red blood cells. Cells were washed once with DMEM medium supplemented with 1% FBS and incubated at 37 °C for 6 h in 96-well, round-bottom microtiter plates (BD Biosciences) in 200 μL of complete DMEM medium with brefeldin A (Golgi Plug; BD Biosciences) at a final concentration of 1 μL/mL. The MHC-I-restricted, E7-specific $CD8^+$ T cell epitope $E7_{49-57}$ (RAHYNIVTF) (GenScript) was used for *in vitro* stimulation at a concentration of 3 μg/mL. After washing, the cells were incubated for 30 min at 4 °C with 100 μL of a 1:100 dilution of FITC-conjugated anti-mouse CD8a mAb (BD Biosciences), washed again with PBS containing 2% FBS, fixed with 4% paraformaldehyde for 20 min and then permeabilized with 0.5% saponin for 20 min at 4 °C. Cells were incubated for 30 min at 4 °C with a 1:100 dilution of a PE-labeled anti-mouse-IFN-γ mAb (BD Biosciences). After washing, the cells were suspended in PBS containing 2% FBS and examined using a FACSCalibur cytometer (BD Biosciences). The data were analyzed by FlowJo software (Tree Star), and the percentage

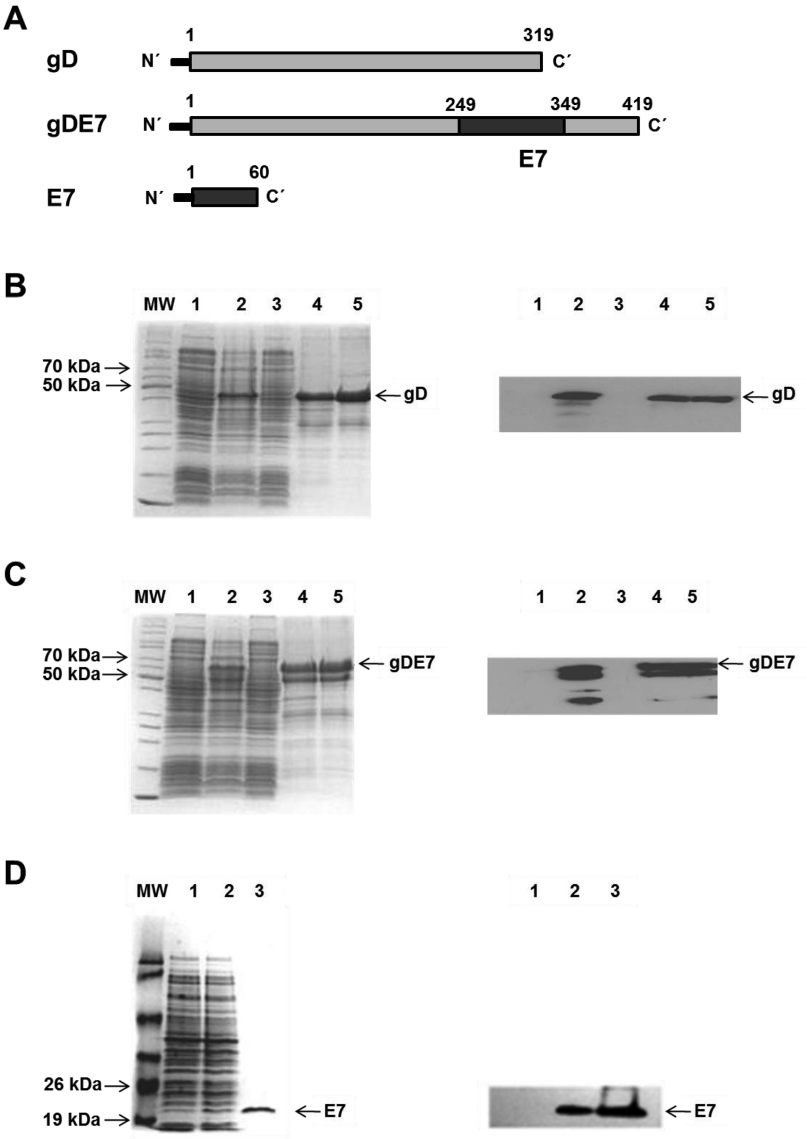

Figure 1. Expression and purification of gD, gDE7 and the truncated E7 protein. (A) Schematic representation of the purified proteins used in the *in vitro* and *in vivo* experiments: gD (consisting of the first 319 amino acids of HSV-1 glycoprotein D), gDE7 (a chimeric protein which contains the entire HPV-16 E7 protein inserted into HSV-1 gD after amino acid 249) and E7 (composed of the first 60 N-terminal amino acids of the HPV-16 E7 oncoprotein). Expression and purification of gD (B), gDE7 (C) and E7 (D) recombinant proteins were monitored by SDS-PAGE (left panels) and immunoblotting (right panels). (B, C) Samples: MW, molecular weight markers; 1, whole-cell extract of noninduced *E. coli* strains; 2, whole-cell extract of the *E. coli* strains after 3 h incubation with IPTG; 3, soluble protein fraction of IPTG-induced *E. coli* strains; 4, insoluble protein fraction of IPTG-induced *E. coli* strains; 5, purified protein following denaturation and refolding steps. (D) Samples: 1, whole-cell extract of noninduced *E. coli* strain; 2, whole-cell extract of recombinant *E. coli* strain after incubation with IPTG for 3 h; 3, purified soluble E7 protein.

of E7-specific $CD8^+$ T cells that stained positive for IFN-γ was determined relative to all $CD8^+$ T cells.

2.8. *In Vivo* Cytotoxicity Assay and Cytokine Dosage. *In vivo* cytotoxicity tests were carried out two weeks after challenge with TC-1 cells, as previously described.[28,29] Briefly, splenocytes collected from naive mice were stained with 0.5 μM or 5 μM of carboxyfluorescein diacetate succinimidyl ester (CFSE) (Invitrogen). Cells labeled with 5 μM CFSE, but not those labeled with 0.5 μM CFSE, were pulsed with 2.5 μg/mL of the $E7_{49-57}$ peptide for 40 min at 37 °C. The same numbers of cells from both populations (2×10^7 cells) were intravenously (iv) injected into mice undergoing the different immunization regimens. One day later, the spleens of inoculated mice were isolated and the splenocytes were examined by flow cytometry. Target cell killing by E7-specific cytotoxic $CD8^+$ T lymphocytes (CTL) was evaluated. The production of cytokines by spleen cells harvested from immunized mice was measured two weeks after TC-1 cell challenge. For *in vitro* stimulation, 1.5 μg/mL of $E7_{49-57}$ peptide or 10 μg/mL of the purified truncated E7 protein was used. Spleen cells were cultured at a final concentration of 10^7 cells/mL in a CO_2 incubator at 37 °C for 72 h. After the incubation time, the supernatants were harvested and stored at −80 °C until the cytokine concentrations were measured by ELISA. ELISAs were performed according to the manufacturer's instructions (BD Biosciences).

2.9. *In Vivo* Activation of Dendritic Cells. Spleen cells from mice intraperitoneally (ip) inoculated with 5 μg of *Salmonella typhimurium* lipopolysaccharide (LPS) (Sigma) or 50 μg of gD or gDE7 were collected 24 h after the immunization, washed twice with RPMI containing 10% FBS and stained with PE-labeled anti-CD11c and either APC-labeled anti-CD86, APC-labeled anti-CD40, APC-labeled anti-CD80, FITC-labeled anti-H2K^b (MHC-I), or FITC-labeled anti-H2IA^b (MHC-II) (BD Biosciences). Labeled cells were suspended in PBS containing 2% FBS and analyzed by flow cytometry (1.5×10^6 events/sample). The data were analyzed using FlowJo software. Groups of mice were inoculated with PBS or heat-denatured gD or gDE7 (50 μg) as controls.

2.10. Statistical Analyses. All data are expressed as the mean $\pm$ SD and represent at least three mice per group. ANOVA and Bonferroni tests were applied, and differences for which $p \leq 0.05$ were considered statistically significant.

3. RESULTS

3.1. Expression and Purification of the Recombinant gD, gDE7 and E7 Proteins. The coding sequences of gD, gDE7 and truncated E7 were cloned into the pET28a(+) expression vector, and the resulting plasmids, pETgD, pETgDE7 and pETE7, were introduced into *E. coli* BL21 (DE3) host cells, generating the recombinant strains BL21gD, BL21gDE7 and BL21E7, which expressed gD, gDE7 and E7 respectively (Figure 1A). Whole cell extracts of the recombinant strains were cultured in the presence of IPTG for 3 h and analyzed by SDS−PAGE and Western blot (Figure 1B−D). The recombinant gD protein had a molecular mass of approximately 44 kDa, as predicted based on the amino acid sequence (Figure 1B). However, the gDE7 protein, with a predicted molecular mass of 55 kDa, showed altered electrophoretic mobility, and two bands of approximately 55 and 65 kDa were observed (Figure 1C). The same protein showed a single peak with an apparent molecular weight of 55 kDa after size exclusion chromatography (data not shown). This observation is consistent with previous data showing anomalous electrophoretic behavior of the HPV-16 E7 protein when expressed by *E. coli*.[30] Western blots performed with an anti-gD monoclonal antibody (DL-6) confirmed the identity of the 44 kDa band and the 55 and 65 kDa bands, corresponding to gD and gDE7, respectively (Figure 1B,C). The gD and gDE7 proteins were found only in the insoluble fraction of *E. coli* although different attempts to change the induction conditions of the recombinant strains have been tested (data not shown). From 1 L of culture we achieved a total yield of approximately 0.12 g/L for gD and 0.14 g/L for gDE7. Purification of the recombinant gD and gDE7 yielded 0.055 g/L and 0.050 g/L, respectively, of the insoluble proteins. Production of soluble gD and gDE7 was achieved after denaturation of the inclusion bodies and subsequent refolding. After the refolding step, the protein yield dropped to approximately 10 mg/L for both proteins. The recombinant truncated E7 protein appeared to have a molecular mass of approximately 20 kDa, and its identity was confirmed by Western blot using an anti-E7 monoclonal antibody. Purification steps generated a soluble protein with a final recovery yield of approximately 10 mg/L (Figure 1D).

3.2. Cell-Binding Activity of the Recombinant gD Proteins. Expression of the HVEM receptor on the surface of nonpermeabilized U937 cells was first confirmed by flow cytometry after labeling with three different specific anti-HVEM monoclonal antibodies (CW1, CW3, CW7) and subsequent incubation with FITC-labeled anti-IgG (Figure 2A). As a second step, we determined the ability of recombinant gD or gDE7 to bind the U937 cells. Cells were incubated with 2 μg of each recombinant protein and labeled with a specific anti-gD monoclonal antibody (DL-6) and FITC-labeled anti-IgG. As shown in Figure 2B, both recombinant gD and gDE7 bound to the U937 cells, but gDE7 bound cells more efficiently than gD (40% of cells were labeled with gD and 78% of cells were labeled with gDE7). As a control, the binding assay was performed with the recombinant proteins after heat-denaturation, and no specific binding was detected (Figure 2B). These results indicated that the recombinant gD and gDE7 proteins retained receptor-binding activities that were lost after denaturation of the proteins. In addition, fusion of gD to E7 enhanced the receptor-binding affinity of the recombinant protein relative to the nonfused gD.

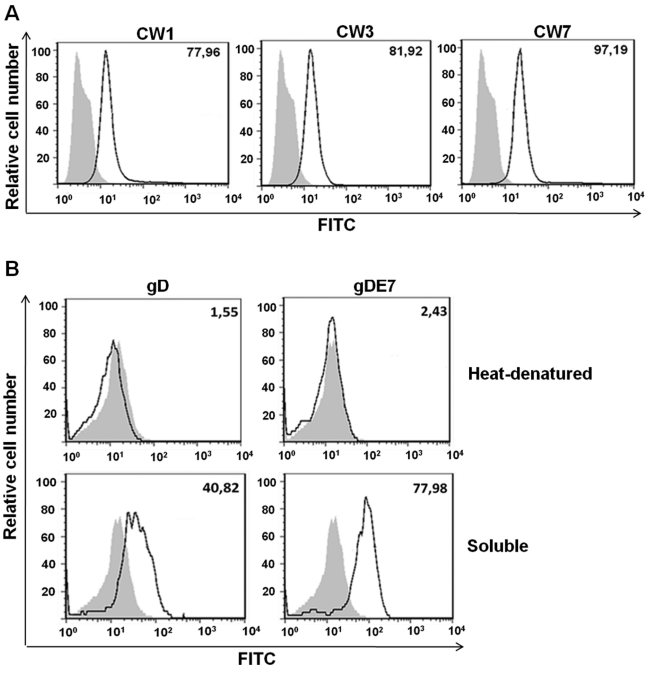

Figure 2. Recombinant gD and gDE7 proteins bind HVEM-expressing U937 cells. (A) Cells were labeled with the anti-HVEM monoclonal antibodies CW1, CW3 and CW7, followed by FITC-labeled anti-IgG. (B) Cells were incubated with heat-denatured or soluble gD and gDE7 proteins for one hour and subsequently labeled with anti-gD monoclonal antibody (DL-6), followed by FITC-labeled anti-IgG. Cells were examined by flow cytometry, and the data were analyzed with the FlowJo software package. Histograms indicated by black lines and gray-shadowed curves show labeled and nonlabeled cells, respectively. Numbers in each panel indicate the percentage of positively stained cells. Results are based on a representative experiment of two independently performed experiments.

3.3. Activation of CD11c$^+$ Cells in Mice Inoculated with gD and gDE7. DC activation is a key step in mounting productive adaptive immune responses mediated by T and B cells. Because DCs are known to express the HVEM receptor, we tested recombinant gD and gDE7 to see whether they promote activation of DC *in vivo*. For that purpose, mice were ip injected with purified gD, gDE7, LPS or PBS, and CD11c$^+$ cells harvested from the spleens of vaccinated mice were monitored for the expression of MHC-I and MHC-II molecules, as well as CD80, CD86 and CD40, by flow cytometry. As shown in Figure 3,

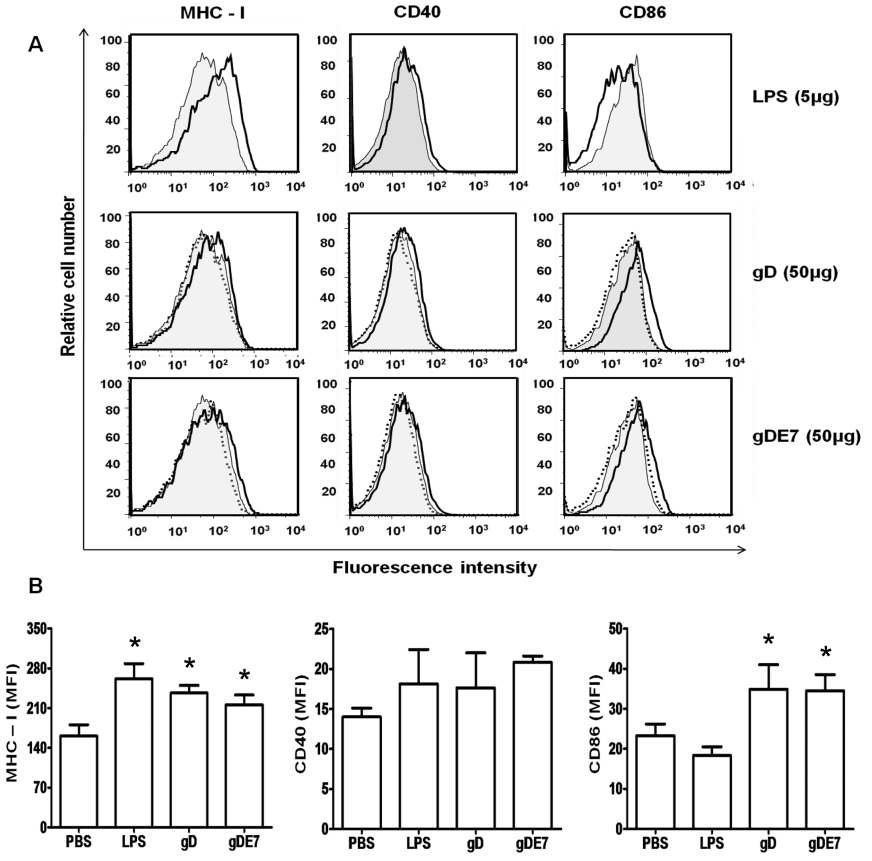

Figure 3. *In vivo* activation of DCs in mice treated with recombinant gD or gDE7. C57BL/6 mice were ip inoculated with LPS (5 μg), gD (50 μg) or gDE7 (50 μg). Twenty-four hours after injection, spleen cells were stained with anti-CD11c, anti-CD86, anti-CD40, anti-CD80, anti-$H2K^b$, or anti-$H2IA^b$ and analyzed by flow cytometry. Histograms representing samples collected from nontreated mice are indicated by lines outlining gray-shadowed curves. Samples collected from mice undergoing the different treatments are represented by curves outlined in black. Samples collected from mice inoculated with denatured recombinant proteins are represented by curves delimited by dotted lines. The expression of surface markers was analyzed by acquiring 1.5×10^6 events. (A) Histograms representing cells gated for CD11c+ expression. (B) Values expressed as mean fluorescence intensity. $^*p \leq 0.05$ compared with nontreated mice. The experiment was repeated twice, and the results represent the mean of three mice $\pm$ SD per group.

$CD11c^+$ cells harvested from mice inoculated with gD, gDE7 or LPS expressed increased levels of MHC-I. Moreover, DCs isolated from mice treated with gD or gDE7 showed statistically significant expression of CD86 but not of CD40, when compared with cells harvested from mice that received LPS or nontreated animals (Figure 3B). Control experiments carried out with heat-denatured gD or gDE7 did not induce significant differences in the expression of the coactivator markers relative to nontreated mice (Figure 3A). No alteration in the expression of MHC-II and CD80 molecules was detected in mice treated with gD or gDE7 (data not shown). These results indicate that the recombinant gDE7 protein and, to a lesser extent, the recombinant gD protein promote the activation of DCs *in vivo*.

3.4. Effect of gD Fusion on Induced Antibody and Th Responses. Because the recombinant gDs proved to be effective for the *in vivo* activation of murine DCs, we evaluated the induced antibody responses directed toward a target antigen (E7) to see whether they would be increased following administration with gD, either as a coadministered adjuvant or as a genetically fused hybrid protein. As indicated in Figure 4A, no relevant differences were detected in the anti-gD IgG titers in sera from mice immunized with gD, gD plus E7 or gDE7. Similarly, there was a small, but not statistically significant, difference in the anti-E7 IgG titers in mice immunized with two or more doses of gDE7 compared with mice immunized only with E7 (Figure 4B). In contrast, we observed a clear shift in the T helper cell response of mice immunized with gDE7, as inferred by the anti-E7 IgG subclass distribution, when compared with mice immunized with E7 or gD plus E7. Sera from mice immunized with gDE7 showed an IgG1/IgG2c subclass ratio of 1.2, while mice immunized with gD plus E7 or only E7 showed IgG1/IgG2c ratios of 643 and 653, respectively (Figure 4C). Similar responses were observed when the E7-specific cytokine secretion profiles were examined. Spleen cells from mice immunized with gDE7 showed high levels of IFN-γ secretion and no detectable IL-10 when spleen cells from vaccinated mice were stimulated with the recombinant E7 protein, whereas cells harvested from mice immunized with E7 secreted high levels of IL-10 and no detectable IFN-γ (Figure 4D). These results indicate that fusion of gD to the E7 protein did not affect the immunogenicity of the antigen with regard to the production of antigen-specific antibodies, but there is a clear modulation of the T helper response toward a more biased type 1 immunity pattern.

3.5. Activation of E7-specific $CD8^+$ T Cell Response in Mice Immunized with the Recombinant gD Proteins. The immunomodulatory effects of the recombinant gD protein on the activation of E7-specific $CD8^+$ T cell responses was examined in mice immunized with four doses of gD, gDE7, gD plus E7, or

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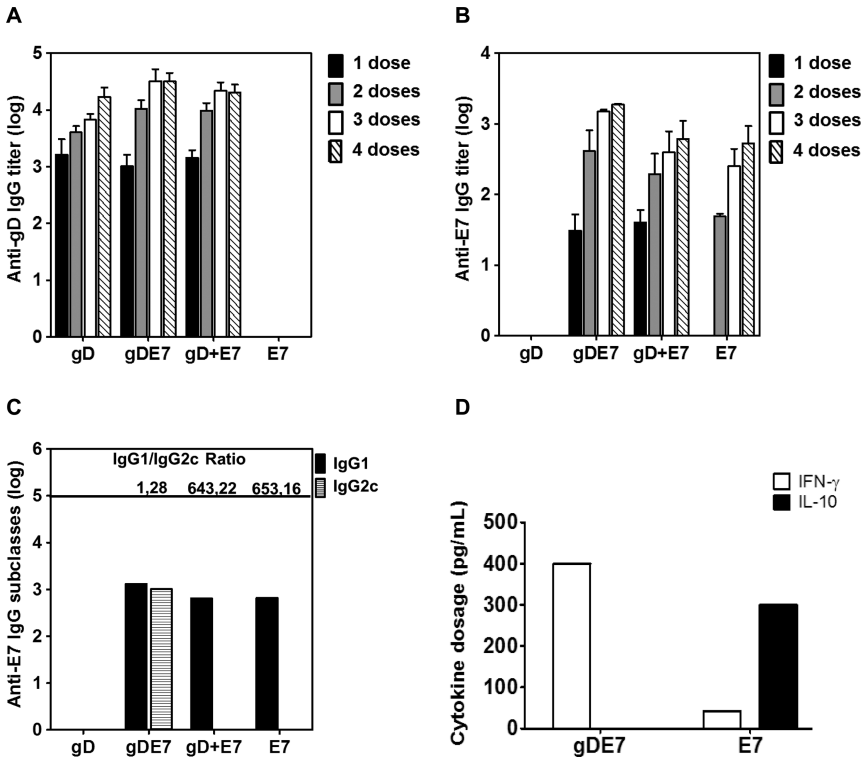

Figure 4. Induced gD- and E7-specific antibody responses and T helper profiles in mice immunized with recombinant E7. (A) gD-specific IgG response elicited in mice immunized with one to four doses of the different vaccine formulations. (B) E7-specific IgG responses elicited in mice immunized with one to four doses of the different vaccine formulations. Serum samples were collected one week after each dose. (C) Anti-E7 IgG subclass responses. Sera used in these test were collected one week after the fourth immunization dose. (D) IFN-γ and IL-10 production detected in the supernatant of spleen cells harvested from vaccinated mice two weeks after TC-1 cell challenge following stimulation with 10 μg purified E7 protein for 72 h. The experiment was repeated twice. The results represent the mean of five mice $\pm$ SD per group.

E7 two weeks after challenge with TC-1 cells. As shown in Figure 5A, mice immunized with gDE7, but not those immunized with E7 or gD plus E7, showed increased activation of E7-specific $CD8^+$ T cells when compared with mice immunized only with gD. Similarly, significant IFN-γ production was detected only in supernatants from spleen cells harvested from mice immunized with gDE7 following stimulation with the MHC-I-restricted $CD8^+$ T cell-specific $E7_{49-57}$ peptide (Figure 5B). To evaluate the functional state of the E7-specific $CD8^+$ T cells induced in mice immunized with gDE7, spleen cells from nonimmunized mice were labeled with two different CFSE concentrations. Cells labeled with the highest CFSE concentration were also pulsed with the $E7_{49-57}$ peptide, mixed with the same amount of the $CFSE_{low}$-labeled cells and iv injected into mice undergoing the different immunization regimens. As shown in Figure 5C, all mice immunized with gDE7, but not those immunized with the other vaccine formulations, mounted E7-specific *in vivo* cytotoxic responses, as demonstrated by the reduction in the number of cells labeled with the E7-specific peptide relative to the population of cells not pulsed with the E7-derived peptide.

3.6. Antitumor Effects Elicited in Mice Immunized with gDE7. Induction of E7-specific $CD8^+$ T cell responses is an essential feature of effective anticancer vaccines based on the TC-1 tumor cells.[23] The antitumor effects conferred by vaccination with the gDE7 protein were determined under two different sets of experimental conditions. The preventive antitumor effects were evaluated by vaccinating mice with the different tested vaccine formulations, followed by injection with the TC-1 cells. Therapeutic antitumor effects, on the other hand, were investigated in mice first inoculated with the TC-1 cells and subsequently treated with four vaccine doses. As observed in Figure 6A, only mice immunized with four doses of gDE7 developed complete preventive protection to TC-1 cells (100% survival), while animals immunized with gD, E7 or gD plus E7 developed tumors after injection with the TC-1 cells. Two months after challenge with the TC-1 cells, mice immunized with gDE7 received a second injection of tumor cells in order to evaluate the longevity of the protective antitumor responses. Under such conditions, all of the vaccinated mice were protected during an additional observation period of one month and showed increased activation of IFN-γ-producing $CD8^+$ T cells (Figures 6A and 5D). In an attempt to decrease the number of vaccine doses used in the immunization regimen, the prophylactic antitumor effects induced by gDE7 were determined in animals immunized with lower numbers of vaccine doses, but only animals receiving four doses were fully protected against tumor growth (Figure 6B). Analyses of the therapeutic antitumor effects elicited in mice showed that 30% of the animals vaccinated with 4 doses of gDE7 controlled tumor development (Figure 6C). These results demonstrate that the recombinant gDE7 protein confers antitumor protection effects on vaccinated mice under both prophylactic and therapeutic conditions.

4. DISCUSSION

Cervical cancer is the second most common cause of cancer death in women, and although two preventive vaccines based on capsid proteins are available, the expected benefits for those

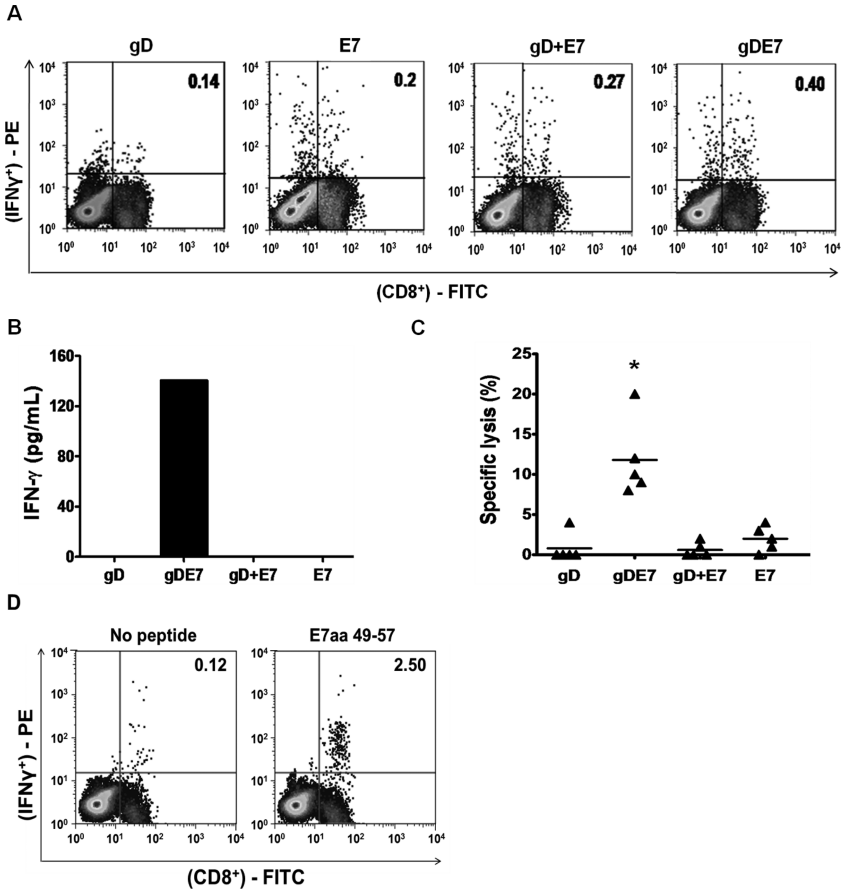

Figure 5. Determination of E7-specific responses mediated by $CD8^+$ T cells. Mice were immunized with four doses of gD, gDE7, gD plus E7 or E7 recombinant proteins and subsequently challenged with 7.5×10^4 TC-1 cells/mouse. Samples were collected two weeks after challenge. (A) Peripheral blood lymphocytes from immunized mice were stimulated *in vitro* with the $E7_{49-57}$ (RAHYNIVTF) peptide, representing the single $CD8^+$ T cell-specific epitope of the E7 protein and used for monitoring of intracellular IFN- γ accumulation. The numbers in the upper right quadrant indicate the percentage of $CD8^+$/IFN-γ^+ lymphocytes relative to the total number of assayed $CD8^+$ T cells. (B) Production of IFN-γ was detected in the supernatant of splenocytes harvested from immunized mice incubated with the RAHYNIVTF peptide. (C) *In vivo* cytotoxic activity of E7-specific $CD8^+$ T lymphocytes from mice immunized with the different vaccine formulations. The result represents the mean percentage of killed target cells labeled with the RAHYNIVTF peptide from five mice per group. * $p \leq 0.05$. (D) Intracellular IFN-γ staining of E7-specific $CD8^+$ T cells performed in PBMCs from mice immunized with gDE7 recombinant protein that were fully protected in the prophylactic TC-1 cell challenge and reinjected with a second dose of cells (7.5×10^4 TC-1 cells/mouse) 60 days later. Samples were collected two weeks after the rechallenge. The numbers in the upper right quadrant indicate the percentage of $CD8^+$/IFN-γ^+ lymphocytes relative to the total number of $CD8^+$ T cells assayed.

already infected or those who cannot afford the high costs of the vaccines are certainly small. Thus, the search for alternative vaccines, particularly those targeting HPV-induced tumors or virus-infected cells, has been a priority for several laboratories around the world.[24,31] Our previous observations showed that DNA vaccines encoding chimeric HPV-16 oncoproteins, particularly E7, genetically fused at a specific site near the C-terminal end of the HSV-1 gD protein increased the activation of E7-specific $CD8^+$ T cells and, more relevantly, enhanced the protective antitumor effects in an experimental murine model.[19] These findings rouse interest in the elucidation of the specific mechanisms behind the strong adjuvant effects of the gD protein on T cell-dependent responses.[20] Nevertheless, questions regarding the gD-mediated adjuvant effects, such as the requirement for endogenous antigen expression and the need to generate hybrid proteins, remained unanswered. In the present study, we tackled these points using purified proteins generated in a prokaryotic expression system. In this scenario we tested a hybrid gDE7 protein, folded or unfolded, and the two isolated proteins, gD and E7. Our results demonstrate that the conformation of the hybrid gD protein and, to a lesser extent, fusion with the target antigen are essential features for the preservation of cell-binding activity, dendritic cell maturation and the activation of antigen-specific T cell responses. Moreover, our results demonstrate that parenteral administration of the chimeric gDE7 without any additional adjuvant was sufficient to induce anticancer protection in mice implanted with TC-1 cells under both preventive and therapeutic conditions. Collectively, these data represent an important step toward a better understanding of the adjuvant effects of gD and may contribute to the future development of a new generation of antitumor vaccines.

Previous observations indicated that the binding of gD to the HVEM receptor occurs through a flexible N-terminal loop that remains partially sheltered by a C-terminal portion of the molecule.[17,18,32] Indeed, modeling of a hybrid gD with a C-terminal fusion with the HIV-1 Gag protein predicted that the C-terminal end would be displaced from the HVEM-binding site.[20] Our results demonstrated that binding of the recombinant

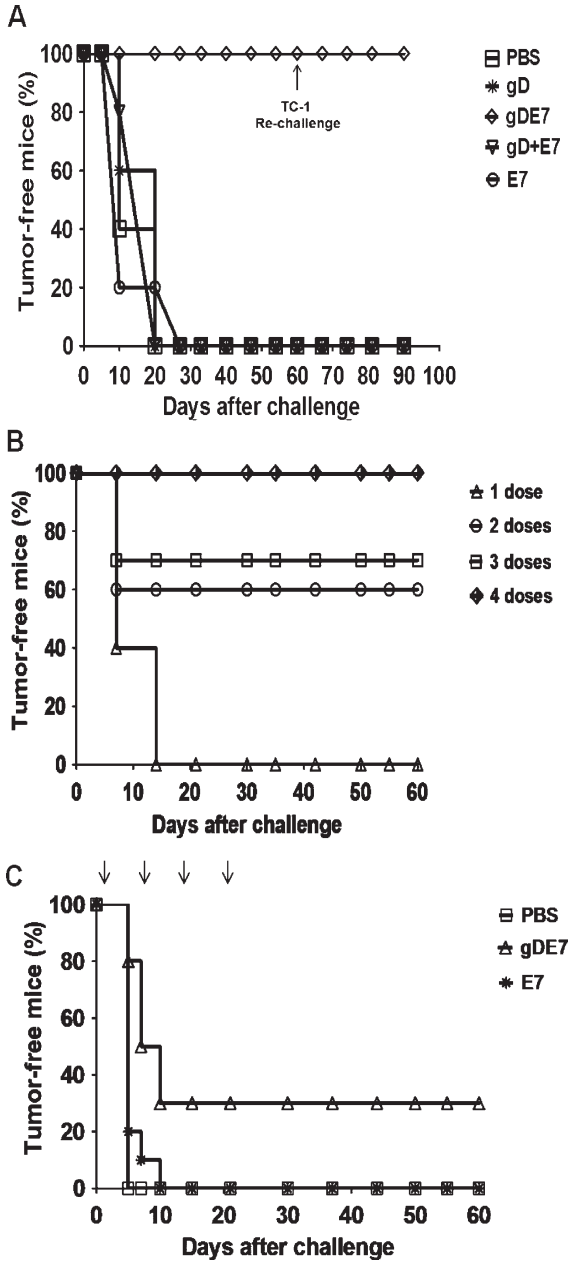

Figure 6. Preventive and therapeutic antitumor effects induced by the recombinant proteins. (A) Preventive protection in C57BL/6 mice sc immunized with four doses of the vaccine formulations administered at weekly intervals. Two weeks after the last dose, mice were challenged with 7.5×10^4 TC-1 cells. Sixty days after the first inoculation with TC-1 cells, the gDE7 group received a second tumor cell challenge (indicated by the vertical arrow). (B) Dose−response curve for tumor-protective responses in mice immunized with gDE7. Mice were immunized with one, two, three or four doses of gDE7 and subsequently inoculated with 7.5×10^4 TC-1 cells. (C) Therapeutic antitumor effects in mice vaccinated with the different vaccine formulations. Mice were inoculated with 7.5×10^4 TC-1 cells and, 24 h later, treated with the immunization regimen based on four vaccine doses administered at weekly intervals (indicated by the vertical arrows). After the TC-1 cell challenge mice were monitored for tumor development by visual inspection and palpation for at least 60 days. Results are based on a representative survival experiments with at least five mice per group.

gDE7 to surface-exposed receptors of the U937 cells, previously shown to express HVEM at the cell surface,[26,27] is better than the binding of the nonfused gD. For both proteins, denaturation reduced the cell-binding activity to negligible levels. The enhanced cell-binding effect may affect the *in vivo* biological activity of gD because this protein has a significantly lower binding affinity to HVEM than other ligands, including BTLA, LIGHT and CD160.[13] These results are also in accordance with previous observations that demonstrated a higher affinity of gD for HVEM when the C-terminal region is deleted or altered.[18] Our results, therefore, confirmed that the fusion of an additional sequence at the C-terminal end of the gD protein enhances its binding to host cell receptors, even without the anchoring at the cell membrane. In fact, our *in vivo* data clearly shown conformation of the gDE7 protein is also essential for the activation of dendritic cells that, otherwise, requires binding and activation the HVEM receptor. It is expected that the enhanced binding activity of the hybrid gD protein would more profoundly affect signaling mechanisms mediated by host cell receptors, particularly, the HVEM receptor. These results also demonstrate that refolding of recombinant gD proteins preserved important conformational determinants of the native protein that are required for proper binding to host cell receptors.

Secondary signals, either costimulatory or coinhibitory, result in the activation of antigen presenting cells, such as dendritic cells, and contribute to the induction of effective adaptive immune responses. Our results demonstrated that *in vivo* administration of purified gD and gDE7 enhanced the expression of MHC-I and CD86 molecules on $CD11c^+$ cells. In fact, *in vitro* evidence has demonstrated that the binding of soluble gD to HVEM results in the activation of NF-κB and a reduction in Fas-induced apoptosis in different cell lineages, features that may affect the penetration of HSV particles into host cells and the subsequent induction of immune responses.[26,27,33] Under our testing conditions, upregulation of MHC-I and costimulatory molecules in mice exposed to gD suggests that blockade of BTLA binding would favor the activation of dendritic cells and T cells, particularly $CD8^+$ T cells, in mice immunized with gD-containing vaccine formulations. In support of these results, previous experiments showed homeostatic expansion of $CD4^+$ and $CD8^+$ T cell populations and enhanced activation of memory $CD8^+$ T cells in BTLA-deficient mice, conditions that would support the formation of a long-lived $CD8^+$ memory T cell pool.[34]

In spite of the fact that HVEM and BTLA are expressed by B and T lymphocytes, available evidence from knockout mice and the administration of soluble agonists indicated that the in vivo modulation of adaptive immune responses mediated by BTLA-HVEM mainly affects T cell responses.[34,35] Our results demonstrated that the gD adjuvant effects observed in mice immunized with the soluble protein were mainly observed in T cells, with only marginal enhancement of the induced antibody responses. A clear shift in T helper responses was observed in mice immunized with gDE7 and subsequently challenged with TC-1 cells, in terms of both the E7-specific IgG subclass response and the cytokine secretion patterns. In contrast to mice with implanted tumors, mice immunized with gDE7 showed a clear type 1 immune response pattern. Administration of gD genetically fused to E7 promoted differential activation of $CD4^+$ T cells without the administration of any additional immune modulator, such as innate immune agonists, which are usually required in immunization regimens with purified proteins. Resting $CD4^+$ T cells express higher BTLA levels and upregulate BTLA expression faster than $CD8^+$ T cells.[35,36] We can conclude, therefore,

that parenteral administration of soluble gDE7 successfully modulated T helper responses that are required for the proper activation and maintenance of cell-mediate immune responses, particularly those mediated by antigen-specific cytotoxic $CD8^+$ T cells.

Immunization of mice with purified gDE7 protein confers antitumor protection in mice challenged with TC-1 cells under both preventive and therapeutic conditions. The preventive antitumor protection in mice immunized with purified gDE7 protein was complete but required at least four sc vaccine doses while the maximal therapeutic antitumor effect was 30% obtained also after four vaccine doses. Our previous results based on DNA vaccines encoding gDE7 showed higher (70%) therapeutic antitumor protection in mice inoculated with TC-1 cells and subsequently immunized with three doses of the vaccine.[21] It is well established that DNA vaccines, as well as attenuated viruses, are better inducers of cellular immune responses, particularly cytotoxic $CD8^+$ T cells, than purified proteins.[1,24,37] It is remarkable, therefore, that administration of purified gD protein genetically fused with the target E7 antigen could elicit a strong activation of cytotoxic E7-specific $CD8^+$ T cells and confer a significant anticancer protection effect in vaccinated mice. These results demonstrate that upon further optimization of the immunization regimen, such as incorporation of adjuvants, higher antigen loads, alternative delivery routes and/or coadministration of immunomodulatory cytokines, additional enhancement of both the T-cell immunogenicity and antitumor effects could be achieved with protein-based vaccine formulations.

The successful clinical experience of HPV-specific therapeutic antitumor vaccines with long synthetic peptides[38] indicates that the way to achieve clinical effective anticancer vaccines may involve the development of protein-based formulation immunogens. The present findings demonstrate that a vaccine containing a hybrid gDE7 protein has some of the features required to generate an effective anticancer vaccine without the inherent risks of attenuated virus or even DNA vaccines. However, additional improvements and further testing are clearly needed before the clinical potential of the proposed vaccine strategy could be evaluated. Issues like pre-existing immunity, particularly against the gD component, and requirement of formulations capable of inducing more robust anticancer responses need further testing and will certainly receive attention in future studies.

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Author Contributions

‡Both authors contributed equally to the present study.

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