Herpes simplex virus glycoprotein D targets a specific dendritic cell subset and improves the performance of vaccines to human papillomavirus-associated tumors

Bruna F. M. M. Porchia[1], Ana Carolina R. Moreno[1], Rodrigo N. Ramos[2], Mariana O. Diniz[1], Laís Helena T. M. de Andrade[1], Daniela S. Rosa[3], José Alexandre M. Barbuto[2], Silvia B. Boscardin[4], Luís Carlos S. Ferreira[1*].

[1] Department of Microbiology, Institute of Biomedical Sciences, University of São Paulo, São Paulo, Brazil. [2] Department of Immunology, Institute of Biomedical Sciences, University of São Paulo, São Paulo, Brazil. [3] Department of Microbiology, Immunology and Parasitology, Federal University of São Paulo-UNIFESP, São Paulo, Brazil. [4]Department of Parasitology, Institute of Biomedical Sciences, University of São Paulo, São Paulo, Brazil.

Running tittle: **DC targeting and antitumor effects of a gD-based vaccine**

Keywords: glycoprotein D, HPV, cancer, dendritic cells, vaccine

Grant Support: BFMM Porchia was a fellow from Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP), grant 2011/20917-0; ACR Moreno was a fellow from FAPESP, grants 2015/16505-0 and 2016/00708-1 and from Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), grant 560713; RN Ramos was a fellow from FAPESP, grant 2011/08905-7; MO Diniz was a fellow from FAPESP, grant 2011/51218-0; LHTM de Andrade was a fellow from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), grant 560486/2010-3; DS Rosa was a fellow from FAPESP, grant 2014/15061-8; SB Boscardin was a fellow from FAPESP, grant 2013/11442; JAM Barbuto was a fellow from FAPESP, grant 2009/54599-5; LCS Ferreira was a fellow from CNPq, grant 520931/1996-3.

***Corresponding author:** Luís Carlos de Souza Ferreira; e-mail: lcsf@usp.br;
Av. Prof. Lineu Prestes, 1374, São Paulo – SP, Brazil; Phone: +55 11 30917356.

Abstract

Cervical cancer is a major public health problem and one of the leading causes of cancer deaths in women. Virtually all cases of cervical cancer, as well as a growing share of anal and head/neck tumors, are associated with human papillomavirus (HPV) infection. Despite the effectiveness, the available prophylactic vaccines do not benefit women with cervical lesions or cancer. Therefore, the search of new immunotherapeutic approaches to treat HPV-induced tumors is still a priority. The present study characterizes a therapeutic antitumor vaccine based on the genetic fusion of the Herpes simplex virus-1 (HSV-1) glycoprotein D (gD) with the E7 oncoprotein from HPV-16 (gDE7). Two subcutaneous doses of gDE7, admixed with poly (I:C), conferred complete and long lasting therapeutic antitumor protection to mice previously challenged with tumor cells expressing the HPV-16 oncoproteins. The vaccine induced multifunctional E7-specific $CD8^+$ T cells with cytotoxic activity and effector memory phenotype ($CD44^+$ $CD62L^{low}$). Additionally, gDE7 admixed with poly (I:C) vaccination controlled the expansion of tumor-induced regulatory T cells and myeloid-derived suppressor cells (MDSCs). More importantly, gDE7 activated mouse $CD11c^+$ $CD8\alpha^+$ and human $BDCA3^+$ dendritic cells (DCs), specialized in antigen cross-presentation to $CD8^+$ T cells, under *in vitro* conditions. These results indicated that the activation of a specific DC population, mediated by gD, improved the antigen-specific immune responses and the therapeutic performance induced by antitumor vaccines. These results open perspectives for the clinical testing of gDE7-based vaccines under the concept of active immunization as a tool for the therapeutic control of cancer.

Introduction

Since the mid-70s, many studies have shown that HPV is present in virtually all clinical cases, being considered the etiologic agent of cervical cancer (1–3). Despite the high efficacy in preventing HPV infection, prophylactic vaccines do not benefit women with precursor lesions or cancer (4). Therapeutic vaccines aim to control the growth of HPV-induced lesions/tumors by activation of cell-based immune responses, particularly tumor-infiltrating cytotoxic $CD8^+$ T cells (5,6). A few other antitumor vaccines evaluated under clinical conditions demonstrated encouraging results regarding induction of immune responses, such as generation of specific antibodies and T cell responses to HPV antigens.(7–10). However, the complete regression of HPV-induced lesions/tumors by therapeutic anti-tumor vaccines, particularly those at more advanced stages, is still an unmet goal. Thus, the discovery of effective and safe therapeutic approaches may radically change the way cervical cancer is currently treated.

In this scenario, our group has developed two therapeutic vaccine strategies to control HPV-16-associated tumors based either on naked DNA (11) or purified recombinant protein (12). Both strategies are based on the expression of a hybrid protein generated after the genetic fusion of HSV-1 gD with HPV-16 E7 oncoprotein (gDE7). Regarding the vaccine formulation based on the purified recombinant protein, our previous observations demonstrated that repeated immunizations with the non-adjuvanted gDE7 protein conferred partial therapeutic antitumor protection to mice transplanted with tumors cells expressing the HPV-16 E6 and E7 oncoproteins (TC-1) (12). The therapeutic antitumor protection mediated by vaccination was linked to the adjuvant properties of the gD protein.

In the present study, we showed that the therapeutic antitumor efficacy of the gDE7-based vaccine could be further enhanced by co-administration of the poly (I:C)

adjuvant, inducing a complete and long lasting therapeutic antitumor protection. Furthermore, immunization with gDE7 and poly (I:C) induced infiltration of E7-specific $CD8^+$ T cells into the tumor microenvironment and prevented the expansion of systemic immunosuppressive cell populations (T reg cells and MDSCs). Remarkably, the adjuvant role of gD protein was linked with the activation of a DC subset specialized in antigen cross-presentation to $CD8^+$ T cells, both in mice and humans. The present observations shed light on the cellular mechanisms of the gD-mediated adjuvant effects involved in the induction of a potent antitumor response to HPV-16-associated tumors.

Material and Methods

Mice and TC-1 tumor cell challenge

The animal facility unit of the Department of Parasitology at the University of São Paulo provided female C57BL/6 wild type (WT), C57BL/6 TLR4 knockout (KO) and C57BL/6 $CD4^+$ T cell KO mice. The animal facility unit of the Department of Immunology at the University of São Paulo provided female C57BL/6 $CD8^+$ T cell KO mice. All animal handling, immunization and euthanasia procedures were approved by the ethics committee for animal experimentation (CEUA 147/2011) and followed the standard rules approved by the National Council for Control of Animal Experimentation (CONCEA). TC-1 tumor cell line was kindly provided by Dr. T.C. Wu at Johns Hopkins University, Baltimore, MD, USA, in 2002 (13). Groups of mice were challenged by subcutaneous injection of 0.1 ml of serum-free media containing 7.5 x 10^4 TC-1 cells in the right rear flank of mice. Tumor growth was monitored by visual inspection and palpation two times a week after challenge. Mice were considered as tumor-bearing when tumors became solid and were euthanized when tumors exceeded

10 mm in diameter. For experiments of tumor microenvironment analysis, TC-1 cells were diluted in Matrigel (BD Biosciences) before inoculation.

Antigens and immunizations

The gD, gDE7 and E7 recombinant proteins were expressed and purified as previously described (12). The concentration and the integrity of the antigens were routinely checked by polyacrylamide gels and the molecular weights corresponded to those predicted *in silico*. LPS quantification was performed with the Limulus Amebocyte Lysate kit (Lonza) according to the instructions of supplier. LPS amounts found in gD, gDE7 and E7 protein solutions corresponded to 0.9, 3.2 and 1.2 ng of LPS per µg of protein, respectively. Vaccine formulations contained 30 µg of gDE7 or 10 µg of E7 each dose, suspended in 200µl of apyrogenic saline. Poly (I:C) (InVivogen) was admixed with gDE7 or E7 recombinant proteins (50ug/dose). In most experiments, mice were subcutaneously vaccinated with two doses of vaccine formulations in weekly intervals, starting one day after TC-1 cells transplantation. The therapeutic vaccination protocol was also performed with two doses of gDE7 admixed with poly (I:C) vaccine formulation starting at the following different time points: 1, 3, 5, 7 or 10 days after challenge. To evaluate the impact of gD-pre-existing immunity in the antitumor response, groups of mice were primed with a single dose of non-adjuvanted gD recombinant protein (22.5 µg per mice), challenged with TC-1 cells and immunized with two doses of gDE7 admixed with poly (I:C).

Immune response analysis

To evaluate the kinetics of the $CD8^+$ T cell response, blood samples were collected weekly and cultured with the CD8-specific E7 peptide (^{49}RAHYNIVTF^{57}-

GeneScript) (1.5ug/ml) (14), Brefeldin A (10 μg/ml - Sigma) and IL-2 (5ng/ml - Sigma). After incubation, cells were stained with anti-CD8a – FITC (cat: 553031) and, after fixation and permeabilization, with anti-IFN-γ – PE (cat: 554412) monoclonal antibodies (mAbs). The percentages of $CD8^+$ IFN-γ^+ cells over all $CD8^+$ T cells were determined. To study the functional profile of $CD8^+$ T cells, spleen cells from immunized mice were re-stimulated with an anti-CD28 antibody (2 μg/ml) (cat: 553295), the CD8-specific E7 peptide (1.5 μg/ml) and Brefeldin A- GolgiPlug™ (10μg/ml - BD Biosciences) for 12 h. After the incubation period, cells were stained with anti-CD8a-Pacific Blue (cat: 558106) mAb, fixed and permeabilized using the Cytofix/ Cytoperm™ kit and stained with anti-IL-2-PE (cat: 561061), anti TNF-α-PE-Cy7 (cat: 557644) and anti-IFN-γ-APC (cat: 554413) mAbs. Boolean platform was used to create all possible combinations of cytokines over the $CD8^+$ population. For memory phenotyping assays, spleen cells were stained with the APC-labeled H-2Db E7-specific dextramer (Immudex) and, subsequently, stained with anti-CD8a Pacific Blue (cat: 558106), anti-CD44 PE (cat: 553134) and anti-CD62L PE-Cy5 (cat: 555545) mAb. The number of effector memory (T_{EM} - $CD44^+CD62L^{low}$) and central memory (T_{CM} - $CD44^+CD62L^{high}$) E7-specific $CD8^+$ T cells were determined. For T reg cells analysis**,** **s**pleen cells were cultured with 10 μg/ml of E7 protein for five days (10,15). After incubation, cells were stained with anti-CD4-FITC (cat:553651), anti-CD25-APC (eBioscience) (cat: 17-0251) and anti-Foxp3-PE (eBiosciences) (cat: 12-4771-80) mAbs using a Foxp3 staining buffer kit (eBioscience). For MDSCs analysis, spleen cells were stained with anti-CD11b-FITC (cat: 553310), anti-Gr-1-PE (cat: 551084) and anti-CD45-PercP-Cy5.5 (eBiosciences) (cat: 45-0451) mAbs. The frequencies of $CD25^+$ $FoxP3^+$ cells over all $CD4^+$ T cells and $CD11b^+$ $Gr\text{-}1^+$ over all $CD45^+$ cells were determined. Unless stated differently, all antibodies were from BD Biosciences.

Samples were acquired on a LSR Fortessa flow cytometer (BD Biosciences) and analyzed using the Flow Jo software (Tree Star).

***In vivo* cytotoxicity**

Cells from *naïve* mice were stained with 0.5 or 5 μM of carboxyfluorescein diacetate succinimidyl ester (CFSE - Invitrogen). The 5 μM CFSE-labeled population was also pulsed with 2.5μg/mL of the CD8-specific E7 peptide. Both peptide-pulsed (target) and non-pulsed (control) cells were injected intravenously (10^7 cells each) into mice previously immunized. One day after the transfer of CFSE-labeled cells, splenocytes were harvested and analyzed for CFSE staining by flow cytometry. The percentages of target-specific cell killing were calculated as described elsewhere (16).

Mouse dendritic cells binding and activating assays

$CD11c^+$ cells were positively selected with magnetic beads according to instructions of the kit supplier (Miltenyi Biotec). The DC yield represented over 80% of the total cells recovery at the end of the procedure. The protein-dendritic cell binding assays were performed with isolated DCs from C57BL/6 WT mice ($4x10^5$ cells/well). Recombinant proteins were used in equimolar amounts [gDE7 (3μg/ml), gD (2.25μg/ml), E7 (0.75μg/ml)]. DCs Fcγ receptors were blocked with anti-CD16/CD32 antibodies (cat: 553140). Cells were incubated with the recombinant proteins and, subsequently, treated with purified anti-His-tag IgG mAb (Thermo Fischer) (cat: MA1-21315). After washes, cells were stained with anti-IgG-PE antibodies (Jackson Immuno Research) (cat: 115-116-146) and, subsequently, incubated with anti-CD3e – biotin (cat: 553060), anti-CD19 – biotin (cat: 553784) and anti-CD49b –biotin (cat:553856) mAbs. Finally, after additional washes, cells were stained with anti-CD11c-APC (cat:550261),

anti-MHC-II-BV421 (cat: 562928), anti-CD8a-BB515 (cat: 564422) mAbs and streptavidin-PerCP-Cy5.5 (BD Biosciences; cat: 551419). The $CD11c^+$ $MHC\text{-}II^+$ population was gated and the binding of the recombinant proteins was evaluated through the median of fluorescence intensity (MFI) of PE for the $CD11c^+$ $CD8\alpha^+$ and $CD11c^+$ $CD8\alpha^-$ DCs.

For DCs activation assays, polymixin B (200µg/ml) was previously incubated with LPS (2µg/ml), or gDE7 (60µg/ml), gD (45µg/ml) or E7 (15µg/ml) proteins for 2 h at 37°C (17). Isolated DCs ($4x10^5$ cells/well) were incubated for 48 h with the following combinations: LPS (100ng/ml); polymixin B (10µg/ml); LPS + polymixin B (100ng/ml + 10µg/ml); gDE7 + polymixin B (3µg/ml + 10µg/ml); gD + polymixin B (2.25µg/ml + 10µg/ml) and E7 + polymixin B (0.75µg/ml + 10µg/ml). Cells were stained with anti-CD11c-PE (cat: 553802), anti-MHC-II-FITC (cat: 553605), anti-CD8α-Alexa Fluor 700 (cat: 557959) and anti-CD40-APC (cat: 558695), or anti-CD80-APC (cat: 560016) or anti-CD86-APC (cat: 565479) mAbs. For intracellular cytokine evaluation, DCs were incubated with the Golgi Stop™ reagent (BD Biosciences) for the last 12 h. DCs were fixed, permeabilized and stained with anti-IL-12 APC (cat: 554480) mAb. CD40 and CD86 MFI values are shown from gated $CD11c^+$ $CD8\alpha^+$ and $CD11c^+$ $CD8\alpha^-$ DCs. The percentages of $IL\text{-}12^+$ cells over all $CD11c^+$ $CD8\alpha^+$ or $CD11c^+$ $CD8\alpha^-$ DCs were determined. Unless stated differently, all antibodies were from BD Biosciences. Samples were acquired on a LSR Fortessa flow cytometer (BD Biosciences) and analyzed using the Flow Jo software (Tree Star).

Human peripheral blood DCs and Mo-DCs activation

Blood DCs from healthy donors were separated by double centrifugation procedures with Ficoll- Hypaque and Percoll 51% (GE Healthcare). After washes, blood

DCs and previously differentiated Mo-DCs were incubated with LPS (100 ng/mL); polymixin B (10 μg/ml); LPS + polymixin B (100 ng/ml + 10 μg/ml); gDE7 + polymixin B (3 μg/ml + 10 μg/ml); gD + polymixin B (2.25 μg/ml + 10 μg/ml) or E7 + polymixin B (0.75 μg/ml + 10 μg/ml). The cells were incubated with the Golgi Plug™ (10μg/ml - BD Biosciences) reagent for 6 h and stained with anti-HLA-DR V500 (cat: 561224), anti-CD11c V450 (cat: 560369), anti CD14 APC (cat: 555399), anti BDCA1 PE (Miltenyi Biotec) (cat: 130-090-508) and anti BDCA3 PE-Vio770 (Miltenyi Biotec) (cat: 130-100-217) mAbs. Cells were fixed/permeabilized and stained with the anti-TNF-α FITC (cat: 554512) mAb. The percentages of TNF-α^+ cells over all Mo-DCs, BDCA3$^+$ or BDCA1$^+$ DCs were determined. Unless stated differently, all antibodies were from BD Biosciences. Samples were acquired on a LSR Fortessa flow cytometer (BD Biosciences) and analyzed using the Flow Jo software (Tree Star).

Statistical Analysis

One-way or Two-way ANOVA followed by Bonferroni post test were performed when individual data were compared. Log-Rank tests were employed whenever survival curves were compared. p-values < 0.05 were considered statistically significant. Statistical analysis and graphical representation of data were performed using GraphPad Prism 5.0.

Results

The therapeutic antitumor activity of gDE7 admixed with poly (I:C) correlates with activation of cytotoxic E7-specific CD8$^+$ T cells

We have previously shown that repeated immunizations with non-adjuvanted gDE7 enhanced the E7-specific CD8$^+$ T cell responses and conferred partial therapeutic

antitumor protection in mice transplanted with TC-1 cells (12). In this paper, we sought to improve the therapeutic antitumor effect of the gDE7-based vaccine by co-administration of poly (I:C), an extensively used vaccine adjuvant with clinical trial records (18). Administration of four subcutaneous doses of gDE7 admixed with poly (I:C) resulted in complete antitumor protection to challenges with transplanted TC-1 cells (Supplementary Fig**.** S1A). The same result was achieved in mice immunized with two subcutaneous doses of this vaccine formulation (Fig. 1A). Under the same conditions, administration of two doses of non-adjuvanted gDE7 or purified E7 admixed with poly (I:C) conferred only 20% of therapeutic antitumor protection while no protection was observed in mice immunized with the E7 antigen alone (Fig. 1A). Based on these results, subsequent immunization experiments were performed with the regimen of two subcutaneous doses of the vaccine formulations. The therapeutic antitumor effects of gDE7 admixed with poly (I:C) were also monitored in mice primed up to 10 days after the TC-1 cell transplantation, when tumors were already palpable . As shown in Fig. 1B, all mice immunized with the first dose of the adjuvanted gDE7 up to 7 days after challenge were tumor-free at the end of the follow-up period (60 days). On the other hand, when the interval between tumor cell transplantation and the vaccine priming was delayed 10 days, the therapeutic antitumor protection declined to 40%.

We monitored the kinetics of $CD8^+$ T cell responses until 28 days after challenge. As shown in Fig. 1C, two subcutaneous doses of adjuvanted gDE7 induced the highest frequency of IFN-γ-producing E7-specific $CD8^+$ T cells in PBMC. The response peak was achieved 14 days after the administration of the second dose of the vaccine formulation. Despite the lower frequency, no statistical differences were found between mice immunized with the gDE7 alone and the adjuvanted gDE7. No E7-specific $CD8^+$ T cell response was detected in mice immunized with either E7 or E7 admixed with

poly (I:C). Similar results were detected in mice immunized with four doses of the tested vaccine formulations (Supplementary Fig. S1B).

To further understand the role of the E7-specific $CD8^+$ T cells on the antitumor protective immunity, we measured the *in vivo* cytotoxic activity of these lymphocytes induced by vaccination. The observed results indicated that, despite the similar performance in activation of E7-specific $CD8^+$ T cell responses, mice immunized with gDE7 or gDE7 admixed with poly (I:C) differed in the *in vivo* cytotoxic activities. While mice immunized with adjuvanted gDE7 exhibited a significant lysis of cells pulsed with the $CD8^+$ T cell-specific E7-derived peptide (~ 60%), mice immunized with non-adjuvanted gDE7 showed a lower activation of E7-specific cytotoxic $CD8^+$ T cells (~ 25%) (Fig. 1D). Residual or no *in vivo* cytotoxic activity was detected in mice immunized with E7 alone or E7 admixed with poly (I:C).

To test if the residual LPS content of gDE7 or E7 recombinant proteins could affect the antitumor immunity conferred to mice transplanted with TC-1 cells, C57BL/6 TLR4 KO mice were immunized with the tested vaccine formulations. As previously observed with WT C57BL/6 mice, two subcutaneous doses of adjuvanted gDE7 conferred complete antitumor protection to challenged TLR4 KO mice (Supplementary Fig. S1C). Similarly, immunizations with adjuvanted gDE7 induced the highest frequency of IFN-γ-producing E7-specific $CD8^+$ T cells in TLR4 KO mice (Supplementary Fig. S1D). These results indicated that the presence of residual LPS did not affect the protective immunity conferred by the vaccine formulations.

To further understand the contribution of T cell subsets in the therapeutic antitumor protection, $CD4^+$ T cell KO, and $CD8^+$ T cell KO C57BL/6 mice were challenged with TC-1 cells and subsequently immunized with gDE7 admixed with poly (I:C). $CD4^+$ T cell KO mice immunized with adjuvanted gDE7 showed the same

antitumor protection detected in WT isogenic mice while, in contrast, $CD8^+$ T cell KO mice showed no protective immunity (Supplementary Fig. S1E). This observation is in accordance with previous observations reporting that protective immunity induced in animals immunized with E7-based antitumor vaccine formulation was $CD8^+$ T cells-dependent but $CD4^+$ T cells-independent (19–21). In addition, we also demonstrated that pre-existing immunity to gD, as generally observed in those previously exposed to HSV, did not hampered the therapeutic antitumor effects induced by gDE7 admixed with poly (I:C) (Supplementary Fig. S1F). .

Collectively, these results demonstrated that the genetic fusion of E7 to gD and the combination of poly (I:C) synergistically enhanced the therapeutic antitumor protection to TC-1 cells challenge by activation of cytotoxic E7-specific $CD8^+$ T cells. To further advance on the understanding of cellular mechanisms that support the antitumor effects induced by gDE7 admixed with poly (I:C), we characterized the specific features of the $CD8^+$ T cell populations specifically induced by the vaccine formulation.

Immunization with gDE7 admixed with poly (I:C) induces the differentiation of multifunctional $CD8^+$ T cells with a predominant effector memory phenotype

Recently, it has been suggested that multifunctional $CD8^+$ T cells are a predictive biomarker of therapeutic vaccines aiming the control of high-grade cervical lesions under clinical conditions (9). Multifunctional $CD8^+$ T cells were evaluated by multiparametric flow cytometry 14 days after the second dose of the tested vaccine formulations (Fig. 2A). Immunizations with adjuvanted gDE7 induced higher frequencies of IFN-γ, TNF-α or IL-2-producing $CD8^+$ T cells compared to the other tested vaccine formulations **(**Figs. 2B-D**)**. Besides that, co-administration of poly (I:C)

increased the frequencies of IFN-γ, TNF-α or IL2–producing $CD8^+$ T cells by approximately 4-, 28- and 140-fold, respectively, regarding mice immunized with non-adjuvanted gDE7. Similarly, administration of gDE7 admixed with poly (I:C) increased the frequencies of IFN-γ, TNF-α or IL-2-producing $CD8^+$ T cells by approximately 4-, 4- and 9-fold, respectively, with regard to mice immunized with E7 admixed with poly (I:C) **(**Figs. 2B-D**)**. Boolean combinations of cytokine-producing $CD8^+$ T cells showed that immunizations with gDE7 admixed with poly (I:C) induced high frequency of multifunctional $CD8^+$ T cells capable to produce IFN-γ and TNF-α simultaneously (Fig. 2E). We also observed a significant proportion of $IL\text{-}2^+$ $CD8^+$ T cells from mice immunized with gDE7 admixed with poly (I:C) regarding the other tested vaccine formulations. Multifunctional $CD8^+$ T cells capable to produce all three cytokines were also mainly detected in mice immunized with gDE7 admixed with poly (I:C) (Fig. 2E).

To further understand the differentiation of the E7-specific $CD8^+$ T cell populations involved in the control of tumor development, we determined the phenotype of these T cell populations. Mice immunized with adjuvanted gDE7 elicited an E7-specific $CD8^+$ T cell response with a prevailing cell population with the effector memory phenotype (T_{EM} - $CD44^+CD62L^{low}$) (Fig. 2F). In addition, the frequency of tumor-infiltrating $CD8^+$ T cells was significantly higher in mice immunized with adjuvanted gDE7 than in mice immunized only with gDE7 or the other tested immunization groups (Fig. 2G). Noticeably, the vast majority of tumor-infiltrating $CD8^+$ T cells activated by immunization with adjuvanted gDE7 were E7-specific $CD8^+$ T cells (~80%) (Fig. 2H). In contrast, immunization with E7 admixed with poly (I:C) promoted activation of E7-specific $CD8^+$ T cells characterized by a central memory phenotype (T_{CM} - $CD44^+CD62L^{high}$) (Fig. 2F), with limited effector function and reduced ability to migrate into the tumor microenvironment (Figs. 2G-H). Thus, the

present data demonstrate that the genetic fusion of E7 to gD protein and co-administration of poly (I:C) efficiently activated multifunctional E7-specific $CD8^+$ T cells with an effector memory phenotype, compatible with the ability to promote tumor growth control.

Control of tumor development mediated by gDE7 admixed with poly (I:C) prevents expansion of immunosuppressive cells

Frequently, Treg cells expand in blood and spleen during tumor development and may impair antitumor immune responses at both induction and effector levels (22). We wondered if immunization with the gDE7-based vaccines would affect the immune suppressive mechanisms driven by the tumor cells. Regarding the analysis of splenic Treg cells, sham-treated mice as well as mice treated with poly (I:C), E7 or E7 admixed with poly (I:C) displayed higher frequencies of splenic Treg cells (~12%, 8%, 12% and 11%, respectively). In contrast, mice treated with non-adjuvanted gDE7 or gDE7 admixed with poly (I:C) showed much lower frequencies of Treg cells (~4% and 2.5%, respectively) (Fig. 3 A-B).

MDSCs also proliferate in lymphoid organs and tumor tissues in response to cytokines and other factors secreted by tumor cells (23). Under our experimental conditions, sham-treated mice as well as mice immunized with E7 or E7 admixed with poly (I:C) showed similar frequencies of splenic MDSCs (~6.7%, 6.2% and 5%, respectively). On the other hand, mice immunized with gDE7 or gDE7 admixed with poly (I:C) presented significantly lower frequencies of splenic MDSCs (~2.5% for both immunization groups) (Figs. 3C-D). Administration of poly (I:C) also caused a significant reduction of splenic MDSCs with regard to sham-treated mice. Collectively, these results demonstrated that the control of tumor development mediated particularly

by gDE7 admixed with poly (I:C) vaccine formulation led to the impairment of splenic Treg cells and MDSCs expansion.

gDE7 binds and activates mouse DCs specialized in antigen cross-presentation

To further understand the mechanisms involved with the antitumor effects of gDE7-based vaccines, we evaluated if the recombinant protein could specifically activate DCs, particularly those directly involved with the cross-presentation of antigens to $CD8^+$ T cells. For that purpose, we measured the interaction of the different recombinant proteins with $CD11c^+$ $CD8\alpha^+$ and $CD11c^+$ $CD8\alpha^-$ DCs (Fig. 4A). As shown in Figs. 4B and C, both gDE7 and gD bound with greater affinity to the $CD11c^+$ $CD8\alpha^+$ DCs, which are particularly relevant for the activation of $CD8^+$ T cell populations. In contrast, the purified E7 antigen showed low specific binding to both DC subsets.

The activation of DCs mediated by recombinant proteins promoted up-regulation of CD40 and CD86 expression and IL-12 production (Figs. 4D-F). gDE7 and gD activated $CD11c^+$ $CD8\alpha^+$ DCs more efficiently than $CD11c^+$ $CD8\alpha^-$ cells. As expected, induction of CD40 and CD86 expression on $CD11c^+$ $CD8\alpha^+$ DCs did not occur after exposure to E7 (Figs. 4D-E). The frequency of IL-12-producing $CD11c^+$ $CD8\alpha^+$ DCs increased after incubation with gDE7 and gD (Fig. 4F). To rule out any effect associated with residual LPS present in the protein preparations, we repeated the activation experiment in the presence of excess endotoxin and polymixin B at the same concentration used with the recombinant proteins. The results demonstrated that the amount of polymixin B present in the assays was sufficient to reduce any effect associated with residual LPS (Supplementary Figs. S2A-C). Collectively, these results indicate that the gD component specifically targeted the E7 antigen to the $CD11c^+$ $CD8\alpha^+$ DCs and promoted differential activation of a murine DC subset specialized in antigen cross-presentation.

gDE7 promotes activation of human DCs specialized in antigen cross-presentation

As an additional step toward the clinical testing of the gDE7-based vaccines, we measured the activation of human DCs under *in vitro* conditions. To demonstrate the direct effects of the recombinant proteins on human DCs, $BDCA1^+$ and $BDCA3^+$ DCs were obtained from healthy human donors (Fig. 5A), promptly stimulated with equimolar amounts of E7, gDE7 or gD and analyzed for TNF-α production. *In vitro* differentiated Mo-DCs were also tested in these experiments. As shown in Fig. 5B**,** activation of $BDCA3^+$ DCs mediated by gDE7 and gD was significantly higher than $BDCA1^+$ DCs. Additionally, gDE7 induced higher frequencies of TNF-α-producing $BDCA3^+$ DCs regarding incubation with E7 (Fig. 5B). Considering that the frequency of $BDCA3^+$ DCs in human PBMCs is approximately 10-fold lower than $BDCA1^+$ DCs (Fig. 5A, (24)), our results demonstrate that both gDE7 and gD promote the specific activation of the DCs directly involved with antigen cross-presentation to $CD8^+$ T cells also in humans. Similarly to the results observed in mice, the presence of polymixin B efficiently counteracted the effects associated with contaminant LPS but did not interfere with the effects associated with the recombinant proteins (Supplementary Fig. S2 D). Taken together, these results demonstrated that the antitumor activity of gDE7 could be ascribed to the adjuvant effects mediated by the gD component, which activates a specific DC population involved with antigen cross-presentation to $CD8^+$ T cells both in mice and human samples.

Discussion

In the present study, we evaluated the adjuvant effects conferred by the HSV-1 gD as an antigen delivery platform for the development of therapeutic vaccines against HPV-16-associated tumors. The approach involves the parenteral administration of gDE7 admixed with poly (I:C) to mice previously transplanted with TC-1 cells. The complete therapeutic antitumor effect was achieved after two doses of the vaccine formulation and the protective immunity was ascribed to the activation of a robust cytotoxic E7-specific response mediated by multifunctional and effector memory $CD8^+$ T cells. In addition, the control of tumor development mediated by the gDE7-based vaccine halted the expansion of systemic immunosuppressive cells, such as Treg cells and MSDCs. Of particular relevance was the finding that gDE7 activated a specific DC subset involved with antigen cross-presentation, both in mice and humans, to $CD8^+$ T cells. Altogether, the present study expands the knowledge about the cellular mechanisms of the immune responses induced by gD-based vaccines and further supports the use of this antigen delivery/adjuvant platform in the development of antitumor immunotherapeutic approaches.

Vaccine-induced antitumor protection in the TC-1 tumor cell model strongly correlates with the activation of cytotoxic $CD8^+$ T cell responses (20,25). However, purified E7 protein is poorly immunogenic and usually does not induce an effective $CD8^+$ T cell response capable to control tumor growth in TC-1 transplanted mice (12,19). The genetic fusion of E7 to non-related proteins is frequently applied to enhance the immunogenicity by increasing presentation via class-I/II MHC molecules (26,27). Previous studies performed by our group showed that the genetic fusion of different antigens nearby the C-terminal region of HSV-1 gD improved the T or B cells responses to these antigens (11,12,28–30). The adjuvant role of gD has been correlated to the binding to the herpesvirus entry mediator (HVEM) receptor and the blockage of

inhibitory signals mediated by B and T lymphocyte attenuator (BTLA) and CD160, which are natural HVEM ligands (31). In addition, the gD/HVEM interaction promotes NF-κB activation and cell survival by itself (32,33), modulating a complex multidirectional signaling pathway that result in the activation of T cell responses (34). Our data now indicates that the gD-mediated adjuvant effects may also involve a targeting mechanism to a specific DC subset specialized in antigen cross-presentation to $CD8^+$ T cells.

The murine $CD11c^+$ $CD8\alpha^+$ DCs efficiently cross-present exogenous antigens on MHC class-I molecules to $CD8^+$ T cells (35). The present study demonstrated that gDE7 bound and activated the $CD11c^+$ $CD8\alpha^+$ DCs more efficiently than the $CD11c^+$ $CD8\alpha^-$ DCs. When activated, $CD11c^+$ $CD8\alpha^+$ DCs are committed with IL-12 production and activation of cytotoxic $CD8^+$ T cells (36). Indeed, we demonstrated an enhancement of IL-12 production by $CD11c^+$ $CD8\alpha^+$ cells after incubation with gDE7. In addition, *in vivo* evidences showed that immunization with the gDE7 admixed with poly (I:C) induced a strong antitumor $CD8^+$ T cell response. Collectively, our results demonstrate that gD adjuvant effects involve targeting and activation of a specific mouse DC subset specialized in antigen cross-presentation, a key step on the mounting of antitumor $CD8^+$ T cell response.

Several studies have demonstrated that TLR engagement also improves antigen cross-presentation to $CD8^+$ T cells (37). Shulz and colleagues showed that TLR3 signaling helps the recognition of double-stranded RNA [poly (I:C)] by $CD11c^+$ $CD8\alpha^+$ DCs and enhances antigen cross-presentation to $CD8^+$ T cells (38). As described elsewhere, co-delivery of an antigen and a TLR ligand to the same antigen-presenting cell improves the activation of T cells (39). In our study, the gD-mediated antigen delivery, leading to activation of $CD11c^+$ $CD8\alpha^+$ DCs, and co-administration of poly

(I:C) supports a similar explanation for the synergistic antitumor effect induced by the tested vaccine formulation.

As discussed above, both gDE7 and poly (I:C) activate DCs specialized in antigen cross-presentation, providing all signals required for activation of an effective $CD8^+$ T cells response. Our study demonstrated that immunization with adjuvanted gDE7 induced a strong cytotoxic, multifunctional and effector memory $CD8^+$ T cells response. Activation of such specific $CD8^+$ T cell population has been correlated with the control of HPV-associated tumors based on the capability to migrate to peripheral tissues and to respond to antigen contact by immediate effector functions (40). Indeed, we found a significant amount of tumor-infiltrating E7-specific $CD8^+$ T cells in mice immunized with gDE7 admixed with poly (I:C). This finding explain, at least in part, the strong vaccine-mediated therapeutic antitumor protection and corroborates clinical data showing that $CD8^+$ T cell infiltration positively correlates with lesion regression (5,6).

The presence of HPV-16 oncoproteins-specific T reg cells in lymph nodes and tumor tissues from cervical cancer patients raised the possibility that oncoprotein-based immunotherapies would result in T reg cells expansion and activation (22). Our study showed that the frequency of splenic T reg cells in mice immunized with adjuvanted gDE7 was similar to the frequency found in naïve mice (~2%) (41). This result indicates that immunization with adjuvanted gDE7 had no direct effect on T reg cells expansion, but contributed to the induction of an effective antitumor $CD8^+$ T cell response. In addition to T reg cells, MDSCs also have a direct role on tumor-mediated immunosuppression (20). Similarly, immunizations with gDE7, regardless of co-administration of poly (I:C), impaired the expansion of splenic MDSCs. Altogether, these results indicated that the gD-mediated immune modulatory effects influence

different cell populations affecting both the effector cytotoxic responses and immune suppression mechanisms.

Human BDCA3$^+$ DCs, the counterpart of the mouse CD11c$^+$ CD8α$^+$ DCs (24), produced elevated TNF-α levels compared to Mo-DCs or BDCA1$^+$ subsets when exposed to gDE7. The ability of human BDCA1$^+$ and BDCA3$^+$ DCs in antigen cross-presentation is linked to several factors including the nature of the antigen, the antigen processing pathway and the DC activation status (42). Regarding the antigen processing pathways, antigens delivered to late endosomes are better cross-presented by BDCA3$^+$ DCs, while antigens delivered in early endosomes are similarly cross-presented by both BDCA1$^+$ and BDCA3$^+$ DCs (43). Although not explored in the present study, the identification of the specific intracellular pathways ascribed to gDE7 processing/presentation in combination to TLR-ligands represents a quite interesting topic and deserve further evaluation.

The use of DCs has been critical for the development of effective antitumor strategies (44,45). As demonstrated here, immunization with adjuvanted gDE7 induced strong antitumor protection and promoted the activation of a DC population specifically involved with antigen cross-presentation and activation of CD8$^+$ T cells. Recent studies report that the priming of specific T cell responses occurs in different HPV-induced cancer patients, including cervical cancer (46), head and neck (47) and oropharyngeal squamous cell carcinoma (48). Nonetheless, an effective tumor control requires adequate immunological stimuli that could afford the concomitant activation of cytotoxic responses and control of tumor-induced immunosuppressive mechanisms. Collectively, our data provide basis for an immunotherapeutic approach by using a DC targeting strategy that can impact the way HPV-induced cancer is currently treated.

Acknowledgments

The authors thankfully acknowledge the technical support of Martins EG and Bertelli C. of Vaccine Development Laboratory, University of São Paulo.

References

1. Gissmann L, zur Hausen H. Partial characterization of viral DNA from human genital warts (Condylomata acuminata). Int J cancer. 1980;25:605–9.
2. Dürst M, Gissmann L, Ikenberg H, zur Hausen H. A papillomavirus DNA from a cervical carcinoma and its prevalence in cancer biopsy samples from different geographic regions. Proc Natl Acad Sci U S A. 1983;80:3812–5.
3. Lin K, Roosinovich E, Ma B, Hung C-F, Wu T-C. Therapeutic HPV DNA vaccines. Immunol Res. 2010;47:86–112.
4. Villa LL. HPV prophylactic vaccination: The first years and what to expect from now. Cancer Lett. 2011;305:106–12.
5. Maldonado L, Teague JE, Morrow MP, Jotova I, Wu TC, Wang C, et al. Intramuscular therapeutic vaccination targeting HPV16 induces T cell responses that localize in mucosal lesions. Sci Transl Med. 2014;6:221ra13.
6. Sun YY, Peng S, Han L, Qiu J, Song L, Tsai Y, et al. Local HPV Recombinant Vaccinia Boost Following Priming with an HPV DNA Vaccine Enhances Local HPV-Specific CD8+ T-cell-Mediated Tumor Control in the Genital Tract. Clin Cancer Res. 2016;22:657–69.
7. Bagarazzi ML, Yan J, Morrow MP, Shen X, Parker RL, Lee JC, et al. Immunotherapy Against HPV16/18 Generates Potent TH1 and Cytotoxic Cellular Immune Responses. Sci Transl Med. 2012;4:155ra138-155ra138.
8. Kenter GG, Welters MJP, Valentijn a. RPM, Löwik MJG, Berends-van Der Meer DM a, Vloon a. PG, et al. Phase I immunotherapeutic trial with long peptides spanning the E6 and E7 sequences of high-risk human papillomavirus 16 in end-stage cervical cancer patients shows low toxicity and robust immunogenicity. Clin Cancer Res. 2008;14:169–77.
9. Kim TJ, Jin H-T, Hur S-Y, Yang HG, Seo YB, Hong SR, et al. Clearance of persistent HPV infection and cervical lesion by therapeutic DNA vaccine in CIN3 patients. Nat Commun. Nature Publishing Group; 2014;5:5317.
10. Welters M, Kenter G, Piersma S, Vloon A, Löwik M, Berends-van der Meer D, et al. Induction of tumor-specific CD4+ and CD8+ T-cell immunity in cervical cancer patients by a human papillomavirus type 16 E6 and E7 long peptides vaccine. Clin Cancer Res. 2008;14:178–87.

11. Diniz MO, Cariri FAMO, Aps LRMM, Ferreira LCS. Enhanced therapeutic effects conferred by an experimental DNA vaccine targeting human papillomavirus-induced tumors. Hum Gene Ther. 2013;24:861–70.

12. Porchia BFMM, Diniz MO, Cariri FAMO, Santana VC, Amorim JH, Balan A, et al. 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. Mol Pharm. 2011;8:2320–30.

13. Lin KYKY, Guarnieri FGFG, Staveley-O'Carroll KFF, Carroll KFS, Pardoll DM, Wu TC, et al. Treatment of Established Tumors with a Novel Vaccine That Enhances Major Histocompatibility Class II Presentation of Tumor Antigen. Cancer Res. 1996;56:21–6.

14. Feltkamp MC, Smits HL, Vierboom MP, Minnaar RP, de Jongh BM, Drijfhout JW, et al. Vaccination with cytotoxic T lymphocyte epitope-containing peptide protects against a tumor induced by human papillomavirus type 16-transformed cells. Eur J Immunol. 1993;23:2242–9.

15. Bolpetti A, Silva JS, Villa LL, Lepique AP. Interleukin-10 production by tumor infiltrating macrophages plays a role in Human Papillomavirus 16 tumor growth. 2010;1–13.

16. De Alencar BCG, Persechini PM, Haolla F a., De Oliveira G, Silverio JC, Lannes-Vieira J, et al. Perforin and gamma interferon expression are required for CD4+ and CD8+ T-cell-dependent protective immunity against a human parasite, Trypanosoma cruzi, elicited by heterologous plasmid DNA prime-recombinant adenovirus 5 boost vaccination. Infect Immun. 2009;77:4383–95.

17. Tynan G a., McNaughton A, Jarnicki A, Tsuji T, Lavelle EC. Polymyxin B inadequately quenches the effects of contaminating lipopolysaccharide on murine dendritic cells. PLoS One. 2012;7:1–10.

18. Ammi R, De Waele J, Willemen Y, Van Brussel I, Schrijvers DM, Lion E, et al. Poly(I:C) as cancer vaccine adjuvant: Knocking on the door of medical breakthroughs. Pharmacol Ther. 2015;146:120–31.

19. Chu NR, Wu HB, Wu TC, Boux LJ, Mizzen L a, Siegel MI. Immunotherapy of a human papillomavirus type 16 E7-expressing tumor by administration of fusion protein comprised of Mycobacterium bovis BCG Hsp65 and HPV16 E7. Cell Stress Chaperones. 2000;5:401–5.

20. Cheng WF, Hung CF, Lin KY, Ling M, Juang J, He L, et al. CD8+ T cells, NK cells and IFN-gamma are important for control of tumor with downregulated MHC class I expression by DNA vaccination. Gene Ther. 2003;10:1311–20.

21. Barrios K, Celis E. TriVax-HPV: An improved peptide-based therapeutic vaccination strategy against human papillomavirus-induced cancers. Cancer Immunol Immunother. 2012;61:1307–17.

22. van der Burg SH, Piersma SJ, de Jong A, van der Hulst JM, Kwappenberg KMC, van den Hende M, et al. Association of cervical cancer with the presence of CD4+ regulatory T cells specific for human papillomavirus antigens. Proc Natl Acad Sci U S A. 2007;104:12087–92.

23. Diniz MO, Sales NS, Silva JR, Ferreira LCS. Protection against HPV-16-associated tumors requires the activation of CD8+ effector memory T cells and

the control of myeloid-derived suppressor cells. Mol Cancer Ther. 2016;15:1920–30.

24. Bachem A, Güttler S, Hartung E, Ebstein F, Schaefer M, Tannert A, et al. Superior antigen cross-presentation and XCR1 expression define human CD11c+CD141+ cells as homologues of mouse CD8+ dendritic cells. J Exp Med. 2010;207:1273–81.

25. Liu H, Wu BH, Rowse GJ, Emtage PCR. Induction of CD4-independent E7-specific CD8+ memory response by heat shock fusion protein. Clin Vaccine Immunol. 2007;14:1013–23.

26. Roman LD, Wilczynski S, Muderspach LI, Burnett a. F, O'Meara a., Brinkman J a., et al. A phase II study of Hsp-7 (SGN-00101) in women with high-grade cervical intraepithelial neoplasia. Gynecol Oncol. 2007;106:558–66.

27. Hallez S, Simon P, Maudoux F, Doyen J, No??l JC, Beliard A, et al. Phase I/II trial of immunogenicity of a human papillomavirus (HPV) type 16 E7 protein-based vaccine in women with oncogenic HPV-positive cervical intraepithelial neoplasia. Cancer Immunol Immunother. 2004;53:642–50.

28. Alves AMB, La O, Almeida DF. DNA immunisation against the CFA / I fimbriae of enterotoxigenic Escherichia coli (ETEC). 2001;19:788–95.

29. Lasaro MO, Diniz MO, Reyes-Sandoval A, Ertl HC, Ferreira LCS. Anti-tumor DNA vaccines based on the expression of human papillomavirus-16 E6/E7 oncoproteins genetically fused with the glycoprotein D from herpes simplex virus-1. Microbes Infect. 2005;7:1541–50.

30. Santana VC, Diniz MO, Cariri F a MO, Ventura AM, Cunha-Neto E, Almeida RR, et al. Bicistronic DNA Vaccines Simultaneously Encoding HIV, HSV and HPV Antigens Promote CD8+ T Cell Responses and Protective Immunity. PLoS One. 2013;8:1–10.

31. Steinberg M, Cheung TC, Ware CF. The Signaling Networks of the Herpesvirus Entry Mediator (TNFRSR14) in Immune Regulation. Immunol Rev. 2012;244:169–87.

32. Sciortino MT, Medici MA, Marino-Merlo F, Zaccaria D, Giuffrè-Cuculletto M, Venuti A, et al. Involvement of gD/HVEM interaction in NF-kB-dependent inhibition of apoptosis by HSV-1 gD. Biochem Pharmacol. 2008;76:1522–32.

33. Cheung TC, Steinberg MW, Oborne LM, Macauley MG, Fukuyama S, Sanjo H, et al. Unconventional ligand activation of herpesvirus entry mediator signals cell survival. Proc Natl Acad Sci U S A. 2009;106:6244–9.

34. Lasaro MO, Tatsis N, Hensley SE, Whitbeck JC, Lin SW, Rux JJ, et al. Targeting of antigen to the herpesvirus entry mediator augments primary adaptive immune responses. Nat Med. 2008;14:205–12.

35. Shortman K, Heath WR, Cd T. The CD8 + dendritic cell subset. 2010;18–31.

36. Farrand KJ, Dickgreber N, Stoitzner P, Ronchese F, Petersen TR, Hermans IF. Langerin+ CD8alpha+ dendritic cells are critical for cross-priming and IL-12 production in response to systemic antigens. J Immunol. 2009;183:7732–42.

37. Kurts C, Robinson BWS, Knolle P a. Cross-priming in health and disease. Nat Rev Immunol. 2010;10:403–14.

38. Schulz O, Diebold SS, Chen M, Näslund TI, Nolte MA, Alexopoulou L, et al.

Toll-like receptor 3 promotes cross-priming to virus-infected cells. Nature. 2005;433:887–92.

39. Garaude J, Kent A, van Rooijen N, Blander JM. Simultaneous Targeting of Toll- and Nod-Like Receptors Induces Effective Tumor-Specific Immune Responses. Sci Transl Med. 2012;4:120ra16-120ra16.

40. van Duikeren S, Fransen MF, Redeker a., Wieles B, Platenburg G, Krebber W-J, et al. Vaccine-Induced Effector-Memory CD8+ T Cell Responses Predict Therapeutic Efficacy against Tumors. J Immunol. 2012;189:3397–403.

41. Chen X, Oppenheim JJ, Howard OMZ, Treg T. BALB / c mice have more CD4+ CD25+ T regulatory cells and show greater susceptibility to suppression of their CD4+ CD25 – responder T cells than C57BL / 6 mice. J Leukoc Biol. 2005;78:114–21.

42. Gutiérrez-Martínez E, Planès R, Anselmi G, Reynolds M, Menezes S, Adiko AC, et al. Cross-presentation of cell-associated antigens by MHC class I in dendritic cell subsets. Front Immunol. 2015;6.

43. Cohn L, Chatterjee B, Esselborn F, Smed-Sörensen A, Nakamura N, Chalouni C, et al. Antigen delivery to early endosomes eliminates the superiority of human blood BDCA3+ dendritic cells at cross presentation. J Exp Med. 2013;210:1049–63.

44. Palucka K, Banchereau J. Cancer immunotherapy via dendritic cells. Nat Rev Cancer. 2012;12:265–77.

45. Barbuto JAM, Ensina LFC, Neves AR, Bergami-Santos PC, Leite KRM, Marques R, et al. Dendritic cell?tumor cell hybrid vaccination for metastatic cancer. Cancer Immunol Immunother. 2004;53:1111–8.

46. van Poelgeest MIE, Visconti V V., Aghai Z, van Ham VJ, Heusinkveld M, Zandvliet ML, et al. Potential use of lymph node-derived HPV-specific T cells for adoptive cell therapy of cervical cancer. Cancer Immunol Immunother. 2016;

47. Garcia-Bates TM, Kim E, Concha-Benavente F, Trivedi S, Mailliard RB, Gambotto A, et al. Enhanced Cytotoxic CD8 T Cell Priming Using Dendritic Cell-Expressing Human Papillomavirus-16 E6/E7-p16INK4 Fusion Protein with Sequenced Anti-Programmed Death-1. J Immunol. 2016;196:2870–8.

48. Masterson L, Lechner M, Loewenbein S, Mohammed H, Davies-Husband C, Fenton T, et al. CD8+ T cell response to human papillomavirus 16 E7 is able to predict survival outcome in oropharyngeal cancer. Eur J Cancer. 2016; 67:141–51.

Figure Legends

Figure 1. Coadministration of poly (I:C) enhances the antitumor effects and activation of E7-specific cytotoxic responses in mice immunized with gDE7. **A,** enhanced antitumor effects in mice immunized with gDE7 admixed with poly (I:C). Mice were challenged with $7.5x10^4$ TC-1 cells and immunized with two subcutaneous doses (days 1 and 8 after challenge, indicated by arrows) of gDE7 or E7 proteins admixed or not with poly (I:C). (*) $p < 0.05$ versus all other immunization groups. Data are based on five mice per group from one out of two independent experiments with similar results. **B,** antitumor effects induced by immunization with gDE7 admixed with poly (I:C) are kept even when the interval between transplantation of the TC-1 cells and the first dose is increased. The first immunization dose occurred 1, 3, 5, 7, or 10 days after transplantation of TC-1 cells and the boost was given 7 days after the prime dose. Mice were followed for 60 days for tumor growth assessment. Data are based on five mice per group from one out of two independent experiments with similar results**. C,** induction of IFN-γ-producing $CD8^+$ T cells after immunization with gDE7 or E7 admixed or not with poly (I:C). The frequencies of IFN-γ-producing E7-specific $CD8^+$ T cells were determined in mice challenged with TC-1 cells and immunized with two subcutaneous doses (indicated by arrows) of the different formulations. Data represent the mean ± SD of five mice per group from one out of two independent experiments with similar results. **D,** *in vivo* cytotoxic activity induced in mice immunized with two subcutaneous doses of tested vaccine formulations (days 1 and 8 after challenge). Percentage of cell death mediated by E7-specific $CD8^+$ T cells after injection of 10^7 target cells in mice submitted to different immunization procedures. (*) $p<0.05$, (***)

p<0.001. Data represent the mean of five mice per group from one out of two independent experiments with similar results**.**

Figure 2. Immunization with gDE7 admixed with poly (I:C) induces activation of multifunctional and effector memory $CD8^+$ T cells. **A-E**, immunizations with gDE7 admixed with poly (I:C) induce the differentiation of multifunctional $CD8^+$ T cells. Mice were immunized with two subcutaneous doses of gDE7 or E7 admixed or not with poly (I:C) on days 1 and 8 after challenge. Spleen cells were analyzed by flow cytometry 14 days after the last immunization. **A**, multiparametric flow cytometry of multifunctional $CD8^+$ T cells. The frequencies of IFN-γ **(B)**, TNF-α **(C)** or IL-2 **(D)** single producers $CD8^+$ T cells were depicted. (*) p<0.05; (**) p<0.01; (***) p<0.001. **E**, after gating on $CD8^+$ T cells, Boolean combinations were created using Flow Jo software to determine the frequency of all possible combinations of cytokine production. (*) p<0.05 versus all other experimental groups in IL-2-producing $CD8^+$ T cells; (**) p<0.01 versus all other experimental groups in IFN-γ and TNF-α-producing $CD8^+$ T cells. Data represent the mean ± SD of 4-5 mice per group from one out of two independent experiments with similar results. **F**, administration of gDE7 admixed with poly (I:C) induces the differentiation of effector memory E7-specific $CD8^+$ T cells. Spleen cells from challenged and immunized mice were labeled with an E7 H-2Db-specific dextramer and subjected to phenotypic analysis using CD44 and CD62L T cell markers by flow cytometry. Effector memory ($CD44^+CD62L^{low}$ - T_{EM}) and central memory ($CD44^+CD62L^{high}$ - T_{CM}) $CD8^+$ T cells ratio in mice challenged with TC-1 cells and subsequently immunized with two doses (days 1 and 8 post challenge) of tested vaccine formulations. (***) p<0.001 (•) p<0.05 versus all other experimental groups in T_{EM}. Data represent the mean ± SD of 4-5 mice per group from one out of two independent experiments with similar results. **G** and **H**, immunization with gDE7 admixed with poly (I:C) induces high frequency of tumor-infiltrating E7-specific $CD8^+$ T cells. Mice were challenged with $7.5x10^4$ TC-1 cells suspended in matrigel and were immunized with two subcutaneous doses (days 1 and 8 post challenge) of tested vaccine formulations. Four days after the last dose, mice were euthanized and tumors/matrigel were removed for tumor microenvironment analysis by flow cytometry. **G**, frequencies of tumor-infiltrating $CD8^+$ T cells induced by the tested vaccine formulations. **H**, cells recovered from tumors/matrigel of E7 admixed with poly (I:C), gDE7 and gDE7 admixed with poly (I:C) immunized mice were labeled with an E7 H-2Db-specific

dextramer and the frequencies of tumor-infiltrating E7-specific $CD8^+$ T cells was determined. (***) $p<0.001$ versus all other experimental groups. Data represent the mean ± SD of four mice per group from one out of two independent experiments with similar results.

Figure 3. Control of tumor development mediated by gDE7 admixed with poly (I:C) vaccine formulation prevents expansion of splenic immunosuppressive cells. For immunosuppressive cells analysis, mice were challenged with $7.5x10^4$ TC-1 cells and immunized with two subcutaneous doses (days 1 and 8 after challenge) of gDE7 or E7 admixed or not with poly (I:C). **A** and **B**, spleen T reg cells do not expand in mice immunized with gDE7-based antitumor vaccines. For Treg analysis, spleen cells were incubated with 10 μg/ml of E7 protein for 5 days. After the incubation time, spleen cells were stained with anti-CD4, anti-CD25 and anti-Foxp3 and frequency of Treg cells was determined. Numbers inside plots represent the frequency of $CD25^+Foxp3^+$ cells over all $CD4^+$ T cells of a representative mouse. (**) $p<0.01$.Data represent the mean ± SD of five mice per group from one out of two independent experiments with similar results. **C** and **D**, spleen MDSCs do not expand in mice challenged with TC-1 cells and immunized with gDE7-based antitumor vaccines. Spleen cells from challenged and immunized mice were stained with anti-CD45, anti-CD11b and anti-Gr-1 antibodies and the frequency of MDSCs is depicted. Numbers inside plots represent the frequency of $CD11b^+$ $Gr\text{-}1^+$ over all $CD45^+$ cells of a representative mouse. (**) $p<0.01$. Data represent the mean ± SD of five mice per group of one out of two independent experiments with similar results.

Figure 4. Targeting of gDE7 to mouse $CD11c^+$ $CD8\alpha^+$ DC leads to DC activation. gD and gDE7 recombinant proteins bind with higher affinity to mouse DC subset specialized in antigen cross-presentation. **A**, gating strategy for analysis of gD, gDE7 or E7 binding and activation of isolated mouse DCs. Doublets and $CD3^+/CD19^+/CD49b^+$ cells were excluded from analysis. $CD11c^+MHC\text{-}II^+$ cells were gated and subsequently separated by the expression of $CD8\alpha^+$. **B**, isolated mouse DCs ($4x10^5$ cells/well) were incubated with gD, gDE7 or E7 proteins at equimolar amounts. Protein binding was detected in 10^5 cells using mouse anti-His tag and anti-mouse IgG-PE monoclonal antibodies. **C**, MFI of protein binding on $CD11c^+$ $CD8\alpha^+$ and $CD11c^+$ $CD8\alpha^-$ DCs. (***) $p<0.001$; (•) $p<0.05$ and (•••) $p<0.001$ versus E7 on $CD11c^+$ $CD8\alpha^+$ DCs; (###)

$p<0.001$ versus E7 on $CD11c^+$ $CD8\alpha^-$ DCs. Data represent the mean of triplicate assays ± SD of one out of two independent experiments with similar results. gD and gDE7 recombinant proteins promote activation of mouse DCs specialized in antigen cross-presentation. DCs activation was determined by up regulation of surface molecules and intracellular cytokine production. MFI of CD40 **(D)** and CD86 **(E)** and IL-12 production **(F)** on $CD11c^+$ $CD8\alpha^+$ and $CD11c^+$ $CD8\alpha^-$ DCs. (***) $p<0.001$; (••) $p<0.01$ and (•••) $p<0.001$ versus E7 on $CD11c^+$ $CD8\alpha^+$ DCs; (#) $p<0.05$ versus E7 on $CD11c^+$ $CD8\alpha^-$ DCs. (★) $p<0.05$ iDC. Data represent the mean of duplicate assays ± SD of one out of two independent experiments with similar results. iDC – cells incubated with apyrogenic saline.

Figure 5. gDE7 protein activates human DCs specialized in antigen cross-presentation. **A**, gating strategy for activation analysis of human MoDCs and blood DCs mediated by gD, gDE7 or E7 proteins. The activation of human DCs was induced by equimolar amounts of gD, gDE7 and E7 recombinant proteins and analyzed by TNF-α production **(B)**. Data represent the mean ± standard error of three healthy donors considered as three independent experiments. (*) $p<0.05$ and (**) $p<0.01$. (ns) not statistically significant. DC – cells incubated with apyrogenic saline.

Figure 1.

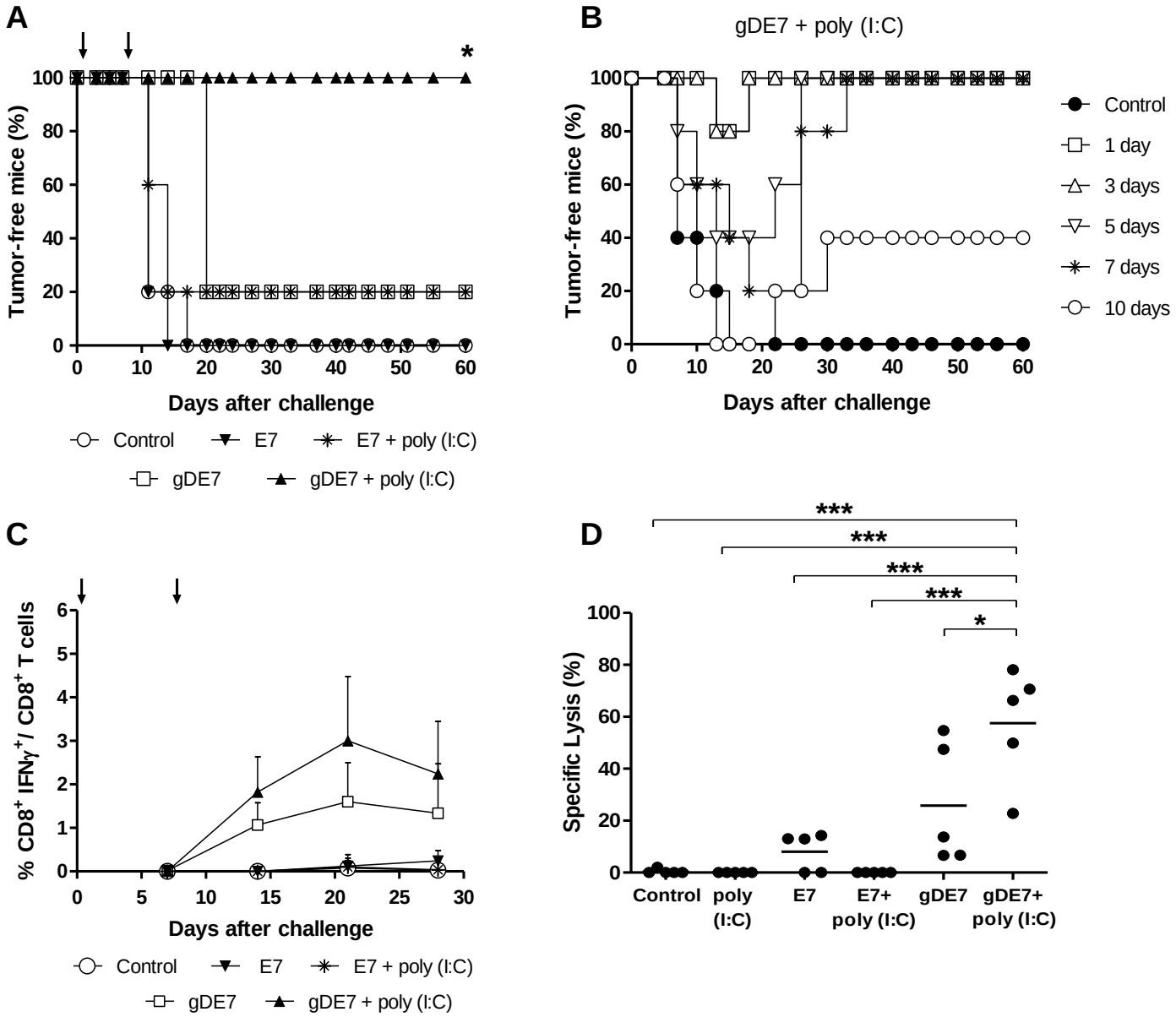


Figure 2.

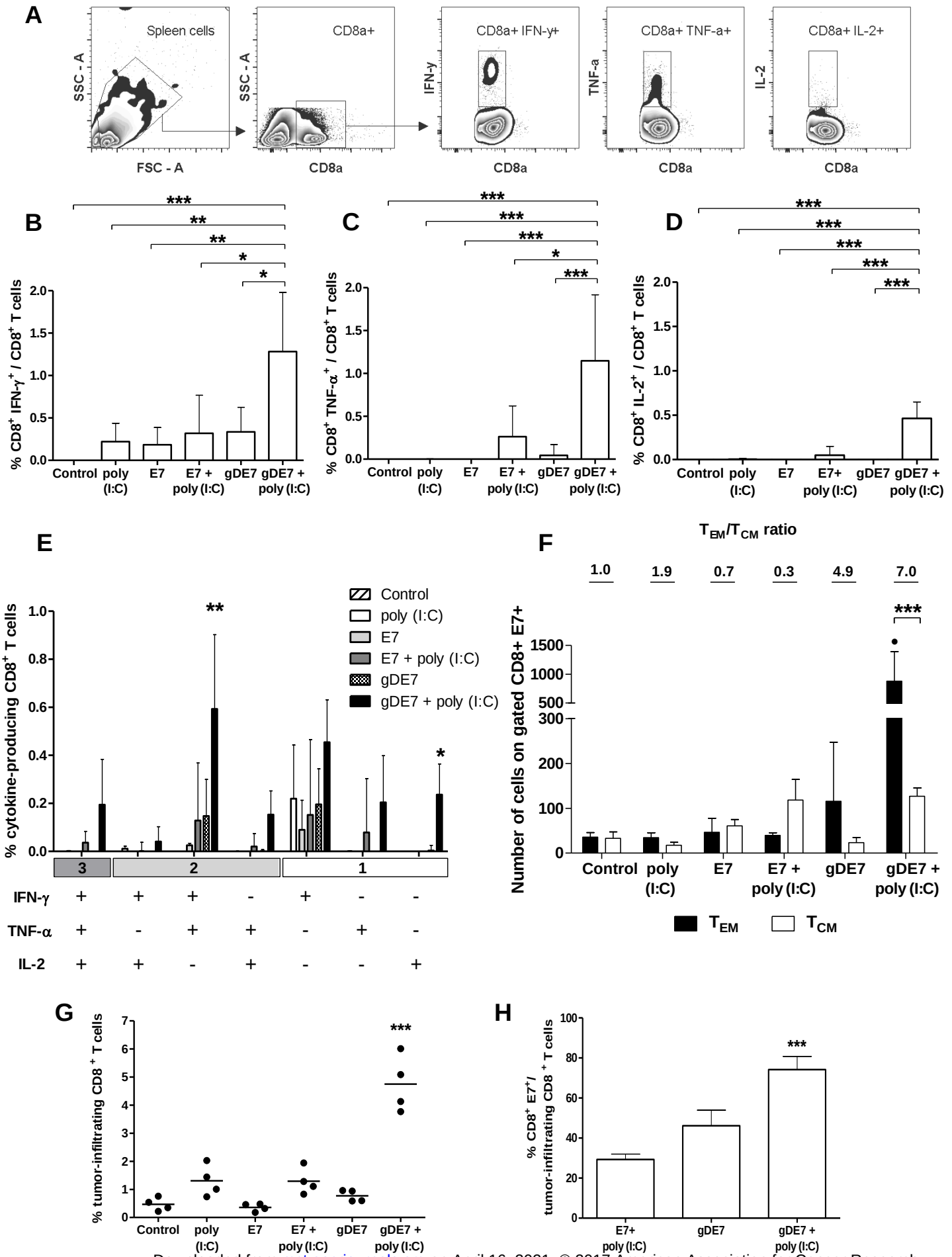


Figure 3.

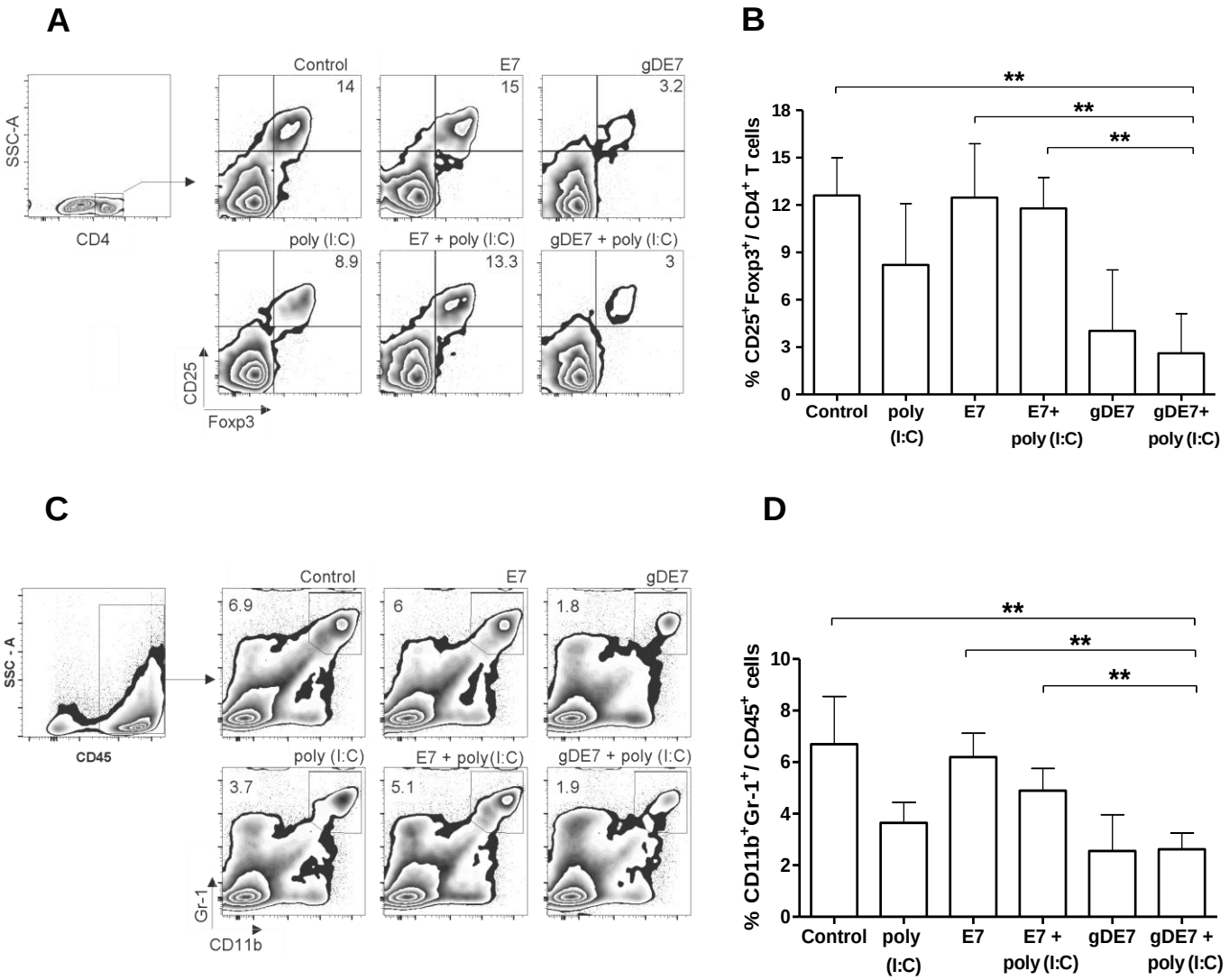
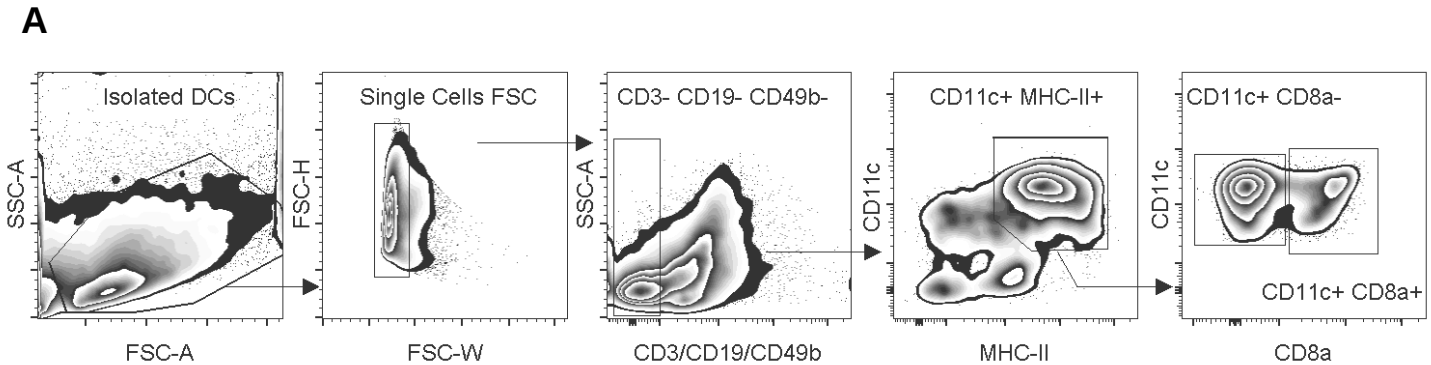
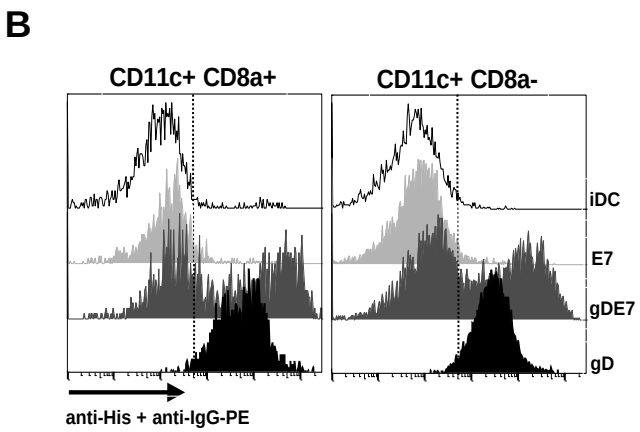
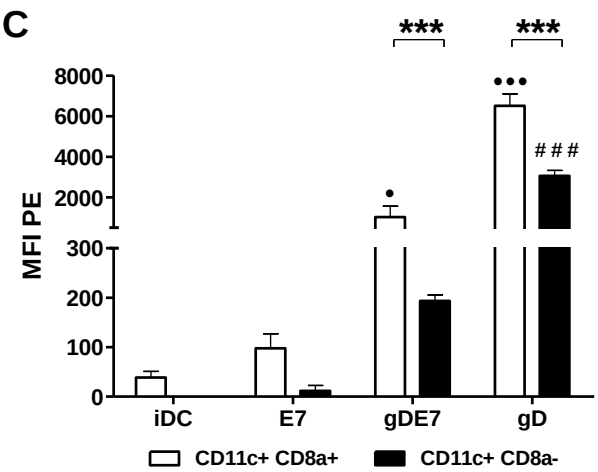
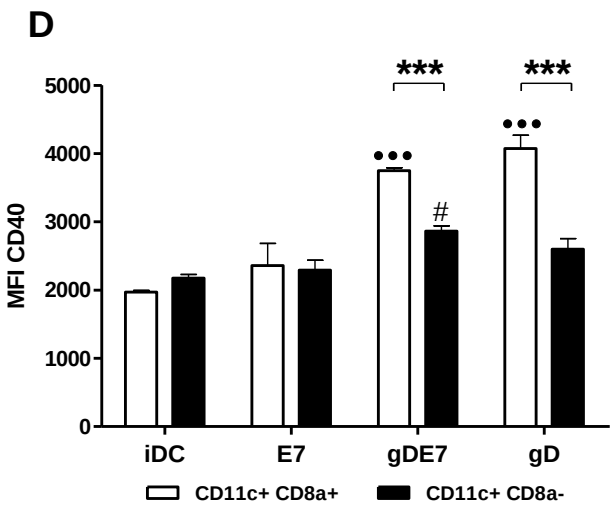


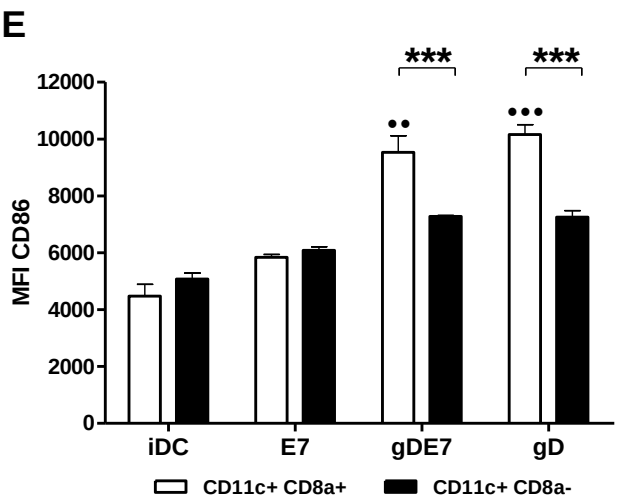
Figure 4.











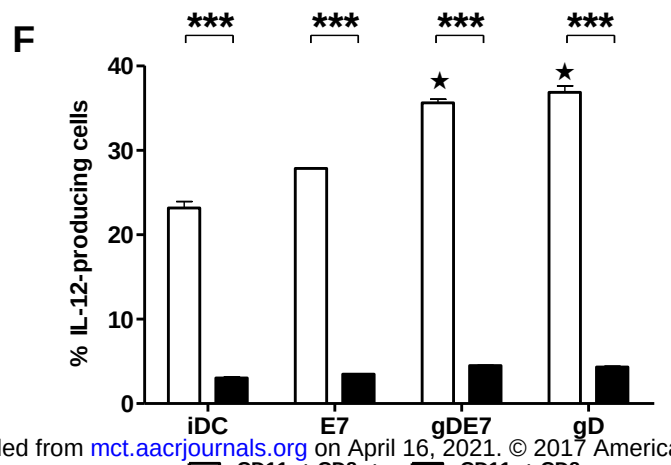
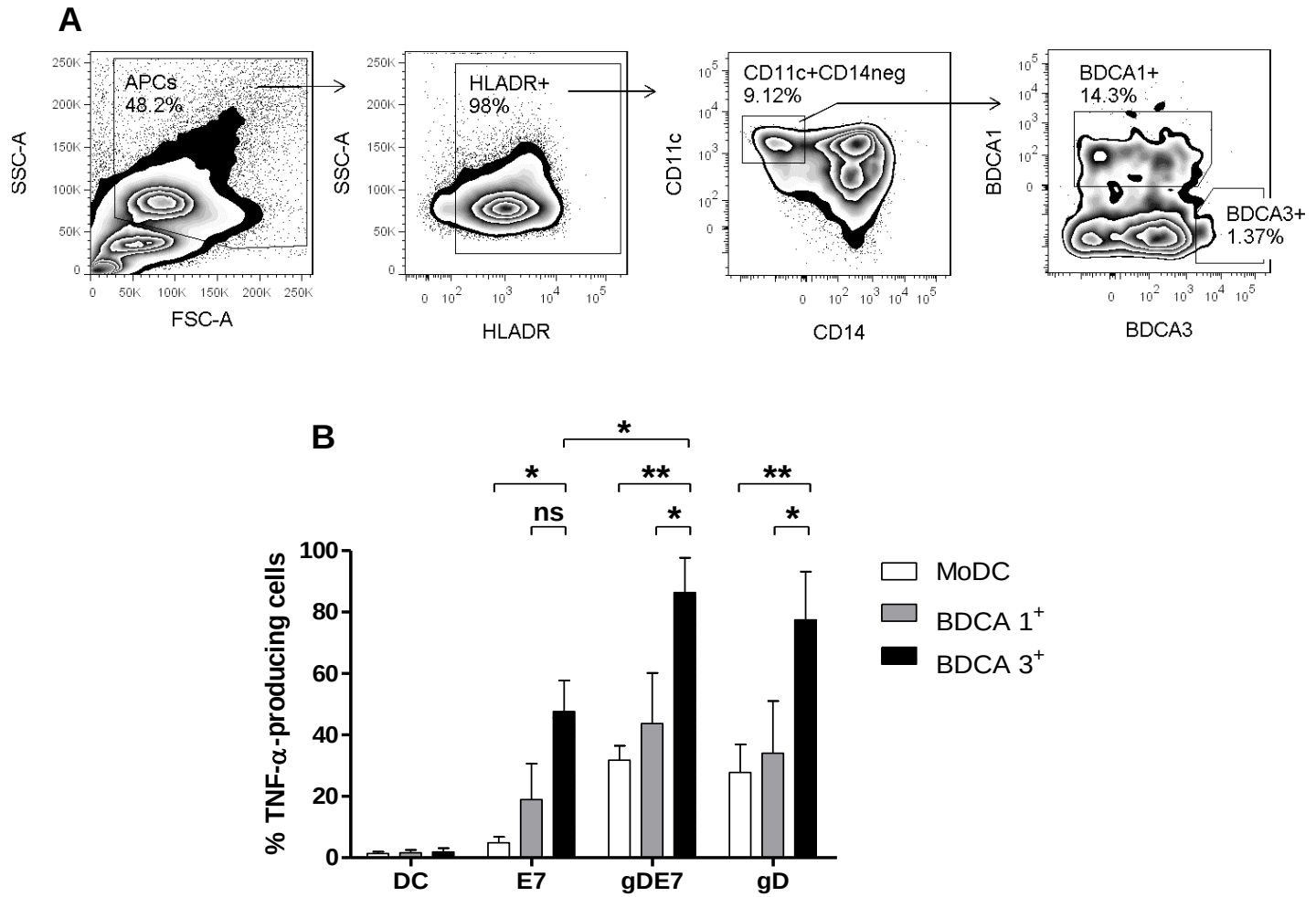


Figure 5.



AACR American Association for Cancer Research

Molecular Cancer Therapeutics

Herpes simplex virus glycoprotein D targets a specific dendritic cell subset and improves the performance of vaccines to human papillomavirus-associated tumors

Bruna F. M. M. Porchia, Ana Carolina R. Moreno, Rodrigo N. Ramos, et al.

Mol Cancer Ther Published OnlineFirst May 18, 2017.