Universidade de São Paulo

Biblioteca Digital da Produção Intelectual - BDPI

Departamento de Microbiologia - ICB/BMM

Artigos e Materiais de Revistas Científicas - ICB/BMM

2013

Bicistronic DNA vaccines simultaneously encoding HIV, HSV and HPV antigens promote CD8 T cell responses and protective immunity

PLOS ONE

Bicistronic DNA Vaccines Simultaneously Encoding HIV, HSV and HPV Antigens Promote CD8+ T Cell Responses and Protective Immunity

Vinicius C. Santana[1], Mariana O. Diniz[1], Francisco A. M. O. Cariri[1], Armando M. Ventura[1], Edécio Cunha-Neto[2], Rafael R. Almeida[2], Marco A. Campos[3], Graciela K. Lima[3], Luís C. S. Ferreira[1]*

1 Department of Microbiology, Biomedical Sciences Institute, University of São Paulo, São Paulo, Brazil, 2 Laboratory of Clinical Immunology and Allergy-LIM60, Division of Clinical Immunology and Allergy, Department of Medicine, University of São Paulo School of Medicine, São Paulo, Brazil, 3 René Rachou Research Center, Fiocruz, Belo Horizonte, Brazil

Abstract

Millions of people worldwide are currently infected with human papillomavirus (HPV), herpes simplex virus (HSV) or human immunodeficiency virus (HIV). For this enormous contingent of people, the search for preventive and therapeutic immunological approaches represents a hope for the eradication of latent infection and/or virus-associated cancer. To date, attempts to develop vaccines against these viruses have been mainly based on a monovalent concept, in which one or more antigens of a virus are incorporated into a vaccine formulation. In the present report, we designed and tested an immunization strategy based on DNA vaccines that simultaneously encode antigens for HIV, HSV and HPV. With this purpose in mind, we tested two bicistronic DNA vaccines (pIRES I and pIRES II) that encode the HPV-16 oncoprotein E7 and the HIV protein p24 both genetically fused to the HSV-1 gD envelope protein. Mice i.m. immunized with the DNA vaccines mounted antigen-specific CD8+ T cell responses, including *in vivo* cytotoxic responses, against the three antigens. Under experimental conditions, the vaccines conferred protective immunity against challenges with a vaccinia virus expressing the HIV-derived protein Gag, an HSV-1 virus strain and implantation of tumor cells expressing the HPV-16 oncoproteins. Altogether, our results show that the concept of a trivalent HIV, HSV, and HPV vaccine capable to induce CD8+ T cell-dependent responses is feasible and may aid in the development of preventive and/or therapeutic approaches for the control of diseases associated with these viruses.

Introduction

The diseases caused by human immunodeficiency virus (HIV), human papillomavirus (HPV) and herpes simplex virus (HSV) represent serious public health threats, as they affect millions of people irrespective of economic or social status [1]. The mortality and morbidity associated with HIV or HSV infection were significantly reduced after the discovery and dissemination of anti-viral therapies that reduce viral loads and relieve symptoms in infected people. However, the currently available drugs are not able to eradicate the viruses, and infections with these viruses remain in a chronic latent state and recur after treatment interruption (HIV) or after debilitation of the immune defenses (HSV). Despite decades of intense scientific work and enormous investments, no effective anti-HIV or anti-HSV vaccine is presently available [2]. Regarding HPV, two prophylactic vaccines that are able to induce antibody responses have been shown to confer protection against virus infection and therefore reduce the long-term incidence of HPV-associated tumors [3,4]. However, the impact on the incidence of HPV-associated cancers is expected to be observed only after the widespread use of these vaccines.

Nonetheless, those already infected with high-risk HPV types or afflicted with HPV-associated cancer or neoplastic lesions are not expected to benefit from preventive anti-viral vaccines. Therefore, the development of therapeutic cancer vaccines that target HPV-infected cells is a priority for several research groups [5].

The concept of therapeutic vaccines relies on the fact that the activation of immunological mechanisms leading to cytotoxic responses, particularly antigen-specific CD8+ T cell activation, permanently eradicates virus-infected or tumor cells [6]. Although theoretically sound and technologically feasible, the development of vaccines that efficiently activate antigen-specific CD8+ T cell populations to control the replication of viruses, such as HIV, remains elusive, as dramatically illustrated by the STEP program [7]. Similarly, numerous attempts to develop both prophylactic and therapeutic anti-HSV vaccines have systematically failed, and new insights regarding the immunological control of HSV-1 and HSV-2 infections are eagerly awaited [8,9]. Vaccines targeting the tumors induced by HPV, under both experimental and clinical conditions, stand as the best and most promising examples of the viability of therapeutic vaccines as immunological tools for the control of infectious and degenerative diseases [10–15].

DNA vaccines have been widely used as therapies against tumors and viruses because of their capability to induce antigen-specific CD8^{+} T cell responses as well as their rather simple manipulation [16,17]. DNA vaccines are also amenable to the development of multivalent formulations either by a mixture of plasmids encoding single antigens or by multiple antigens expressed as fused epitopes [18] or proteins derived from the same or different pathogens [19–21]. Multivalent DNA vectors can also be engineered to encode polycistronic transcripts under the control of a single promoter, leading to the simultaneous expression of multiple antigens in transfected host cells [22–26].

We have previously shown that DNA vaccines encoding the HPV-16 E7 oncoprotein genetically fused to HSV-1 glycoprotein D (gD) enhance both the induction of E7-specific CD8^{+} T cell responses and therapeutic/prophylactic anti-tumor effects compared to vaccines encoding the non-fused HPV oncoproteins in mice [15,27,28]. Additional evidence has indicated that these gD-dependent immunological effects, particularly the activation of CD8^{+} T cell responses to bystander antigens, involve the binding of gD to herpes virus entry mediator (HVEM) and the blockage of a co-inhibitory immune mechanism involving the B- and T-lymphocyte attenuator (BTLA) cell receptor [13,29]. In addition, further experimental evidence has indicated that the binding of gD to HVEM not only represents an important step in virus entry but also triggers an NF-kB-activation pathway and inhibits apoptosis in HVEM-expressing cells, such as antigen-presenting cells [30,31].

More recently, the fusion of gD with the influenza virus nucleoprotein further demonstrated that an adjuvant effect on CD8^{+} T cell responses can be achieved with different antigens and reveals the possibility of the development of multivalent anti-virus vaccine formulations that are able to induce prophylactic and/or therapeutic protective immune responses [32]. In the present study, we applied this vaccine technology to develop trivalent DNA vaccines encoding the HSV-1 protein gD genetically fused with both the HPV-16 oncoprotein E7 and the HIV-1 protein p24 by the same transfected cell. To achieve the simultaneous expression of the two hybrid proteins using a single vaccine vector, we employed a bicistronic expression system using an internal ribosome entry site (IRES) sequence [23,24]. Two vaccine vectors were constructed, pIRES I and pIRES II, which expressed the recombinant hybrid proteins under the control of a strong virus promoter (CMV) but differed in the order of the cistrons with respect to the promoter. Mammalian cells transfected with the recombinant vectors simultaneously expressed the HSV, HIV and HPV proteins at the cell surface. More importantly, C57BL/6 or BALB/c mice immunized with the tested vaccines elicited antigen-specific CD8^{+} T cell responses and were protected to challenges with a vaccinia virus expressing the HIV-1 protein Gag and a HSV-1 strain. In addition, vaccine administration could therapeutically eradicate the growth of tumor cells expressing the HPV-16 oncoproteins implanted in C57BL/6 mice. Altogether, the present study demonstrates that the generation of a trivalent HIV, HSV and HPV anti-cancer vaccine is a feasible goal and should be pursued as a potential tool to prevent and/or treat infections with these viruses.

Results

### Generation of Trivalent DNA Vaccines that Simultaneously Encode HIV, HSV and HPV Antigens

The DNA vaccines tested in this study were obtained by amplifying the chimeric HPV-16 E7 or HIV-1 p24 coding sequences, both of which were fused to the HSV-1 gD sequence,

and cloning them into the pIRES vector. This process generated two bifunctional plasmids, pIRES I and pIRES II, which only differ in the order of their cloned chimeric genes (**Figure 1A**). pIRES I carries the gDp24 gene fusion upstream of the IRES sequence and the gDE7 gene fusion downstream of this regulatory sequence. In contrast, the pIRES II vector carries the gDE7 and gDp24 sequences upstream and downstream of the IRES sequence, respectively. To confirm the expression and cellular localization of the encoded hybrid proteins, the recombinant DNA vaccines were introduced into mammalian COS-7 cells, and the encoded proteins were observed by immunofluorescence. The HSV-1 protein gD is a viral envelope glycoprotein that is expressed on the surface of infected cells via a C-terminal anchor sequence [33]. Thus, the recombinant proteins were detected in non-permeabilized cells treated with specific monoclonal (gD) or polyclonal (p24 and E7) antibodies against the target antigens. Cells transfected with pIRES I or pIRES II were simultaneously stained with antibodies against all three viral antigens (**Figure 1B**). Signals corresponding to the three proteins were detected mainly at the surface of the non-permeabilized cells. These results indicate that the hybrid proteins were correctly folded and properly targeted to the cell membrane compartment of the pIRES I- and pIRES II-transfected cells.

pIRES I and pIRES II Vaccination Induces the Activation of Antigen-specific CD8^{+} T Cells and Simultaneous Protective Responses against Gag-expressing Vaccinia Virus and Tumor Cells Expressing HPV-16 Antigens

Mice were immunized with pIRES I and pIRES II and monitored for the activation of E7-specific (in C57BL/6 mice) and p24-specific (in BALB/c mice) CD8^{+} T cells, the key immunological response required for both the control of virus-induced tumors and intracellularly replicating viruses. The CD8^{+} T cell mediated responses were first monitored in the vaccinated mice by detecting the level of antigen-specific IFN-γ^{+} -producing CD8^{+} T cells using both ICS and ELISPOT assays for total spleen cells harvested fourteen days after the last immunization dose. The cells harvested from the vaccinated mice were incubated with synthetic peptides corresponding to the immunodominant HPV-16 E7 and HIV-1 p24 MHC-I-restricted CD8^{+} T cell-specific epitopes for the H-2K[b] and H-2K[d] haplotypes, respectively. Cells stimulated with the H-2K[d]-restricted p24 peptide for 6 h resulted in the activation of p24-specific IFN-γ^{+}/CD8^{+} T cells at frequencies of approximately 1% and 0.6% in BALB/c mice immunized with pIRES I and pIRES II, respectively, as determined by ICS (**Figure 2A**). Following the same stimulation procedure with the p24-specific peptide but evaluating the results with an ELISPOT assay, only the animals vaccinated with pIRES I reached a statistically higher response compared with the control group immunized with the empty DNA vector (**Figure 2B**). The E7-specific IFN-γ^{+}/CD8^{+} T cell responses elicited in the vaccinated C57BL/6 mice were determined using the H-2K[b]-restricted E7-specific peptide. Intracellular IFN-γ staining showed that the animals immunized with pIRES I or pIRES II developed higher E7-specific IFN-γ^{+}/CD8^{+} cell responses compared with mice immunized with the control vector, while only the animals immunized with the pIRES II vector generated positive responses compared with the control group when the responses were analyzed with the ELISPOT assay (**Figure 2C and D**). In addition, the E7-specific responses detected in the mice immunized with pIRES II were statistically higher than those detected in the mice immunized with pIRES I, as monitored by ICS (**Figure 2C**). Collectively, these results indicate that immunization with pIRES I and pIRES II induces
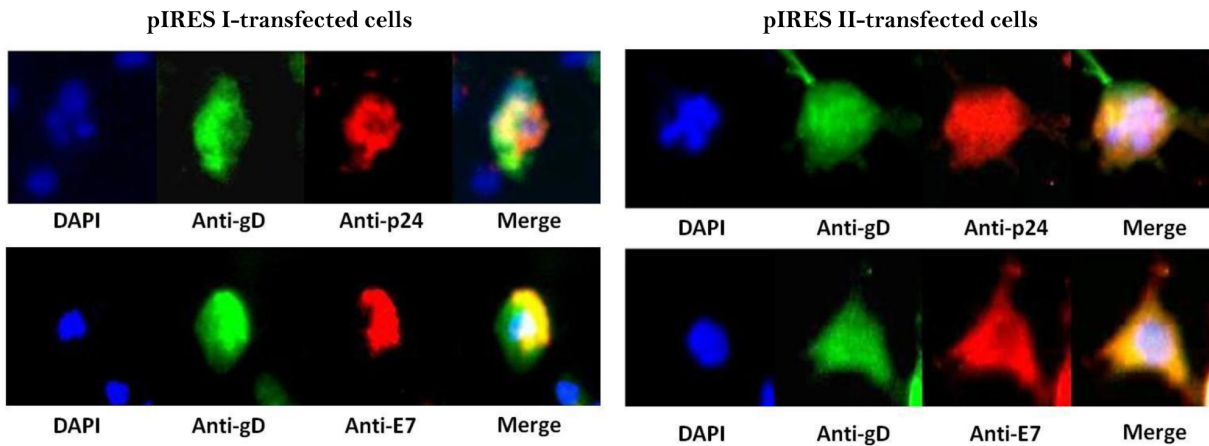
A
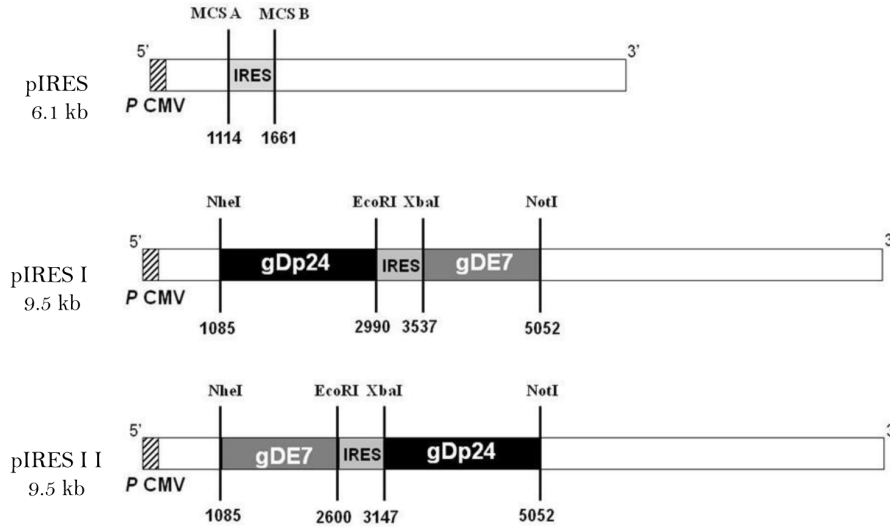
B

Figure 1. Construction of bicistronic DNA vaccines encoding HPV, HIV and HSV antigens for expression in mammalian cells. (A) Schematic linear representation of the trivalent DNA vaccines. pIRES I and pIRES II contain gDp24 and gDE7 chimeric gene fusions, which are inverted with regard to the CMV promoter and IRES sequence. The empty vector pIRES Ø was used as a control. The nucleotide numbers corresponding to the IRES sequence and the cloned chimeric genes are indicated. (B) In vitro expression of the chimeric proteins encoded by pIRES I (left panels) and pIRES II (right panels). Non-permeabilized pIRES I- or pIRES II-transfected COS-7 cells were labeled with antigen-specific antibodies for the simultaneous detection of the HSV-1 protein gD and the HIV-1 protein p24 or the HPV-16 oncoprotein E7. Green, gD; red, p24 or E7; yellow, co-localization of gD with p24 or E7; blue, DAPI nuclear staining.
doi:10.1371/journal.pone.0071322.g001

p24- and E7-specific CD8$^+$ T cell responses in mice, but higher E7-specific IFN-γ^+/CD8$^+$ T cell responses are present in mice immunized with the vector in which the E7/gD fusion is encoded by the first cistron (pIRES II).

To further demonstrate that the antigen-specific activation of CD8$^+$ T cells was functional in the vaccinated mice, we measured the in vivo antigen-specific cytotoxic responses elicited in mice immunized with pIRES I or pIRES II. Splenocytes harvested from non-vaccinated mice were surface labeled with both a fluorescent label (CFSE) and either the MHC-I restricted p24- or E7-specific peptide. The labeled cells were intravenously introduced into vaccinated mice, which were monitored 24 h later for the specific lysis of the labeled cells in their spleens. The relative reduction in

the level of peptide-labeled cells compared with cells labeled only with CFSE was indicative of antigen-specific CD8$^+$ T cell-dependent cytolytic activity. Mice immunized with pIRES I mounted a p24-specific CD8$^+$ T cell-dependent cytotoxic response, while mice immunized with pIRES II showed a statistically significant E7-specific CD8$^+$ T cell-dependent cytotoxic response compared with mice immunized with the empty vector (**Figure 3A and B**). These results further demonstrate that effective antigen-specific CD8$^+$ T cell-dependent cytotoxic responses are induced by the vaccines in which the target gene is expressed by the first cistron of the bicistronic transcript.

As a final step to evaluate the protective immunological status of the mice immunized with the DNA vaccines encoding the HPV
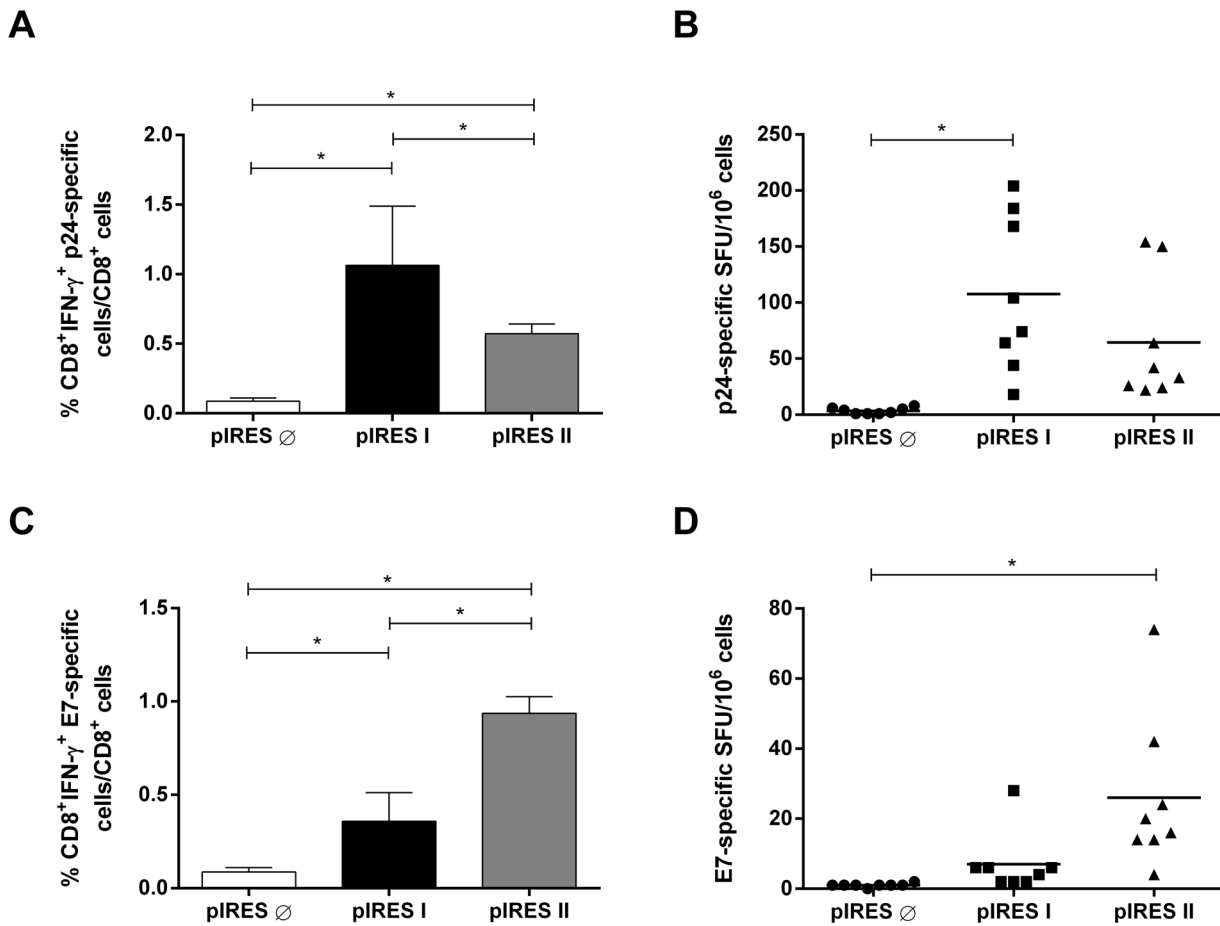
Figure 2. Activation of antigen-specific IFN-γ-producing CD8$^+$ T cell precursors in mice immunized with pIRES I or pIRES II. (A-B) Spleen cells from BALB/c mice spleen cells were stimulated with the MHC-I-restricted p24-specific peptide, and the p24-specific IFN-γ-producing CD8$^+$ T cells were detected by intracellular cytokine staining (A) or ELISPOT assay (B). (C–D) Spleen cells from C57BL/6 mice were stimulated with the MHC-I-restricted E7-specific peptide, and the E7-specific IFN-γ-producing CD8$^+$ T cells were detected by IFN-γ intracellular staining (C) or ELISPOT assay (D). Mice were i.m. immunized with three doses of the DNA vaccines with one week intervals between doses (100 μg/dose). The CD8$^+$ T-cell responses were analyzed two weeks after the last dose. *p<0.05. Data represent the compilation of two independent experiments with four mice per immunization group (n = 8) and results expressed by each animal analyzed. pIRES is the empty vector used as immunization control.
doi:10.1371/journal.pone.0071322.g002

and HIV antigens, we submitted the vaccinated mice to challenges with a recombinant vaccinia virus expressing the Gag protein or TC-1 tumor cells expressing the HPV-16 E6 and E7 oncoproteins. As shown in **Figure 3C**, female BALB/c mice immunized with pIRES I or pIRES II showed significantly less of the recombinant vaccinia virus in their ovaries after challenge with the vAbT141 vaccinia strain (1,000- or 100-fold less compared with the control group immunized with the empty DNA vector, respectively). Similarly, C57BL/6 mice immunized with pIRES I or pIRES II developed protective anti-tumor immunity. All mice challenged two weeks after immunization with pIRES II remained tumor free, while 60% of the mice immunized with pIRES I remained tumor-free after transplantation of the TC-1 cells (**Figure 3D**). Notably, mice immunized with the DNA vaccines developed partial therapeutic anti-tumor immunity. Twenty percent of C57BL/6 mice transplanted with TC-1 cells and subsequently treated with three doses pIRES I remained tumor-free, while 60% of the mice immunized with pIRES II developed a therapeutic anti-tumor response, as they were able to eradicate the TC-1 cells that were previously transplanted into their bodies (**Figure 3E**). Collectively, these results indicate that the DNA vaccines are able to induce functional and protective CD8$^+$ T cell-dependent responses in

vaccinated mice, leading to efficient anti-virus (HIV) and anti-tumor (HPV) effects. In addition, these results indicate that vaccination with these DNA vectors, particularly pIRES II, confers therapeutic immune responses to tumor cells.

Vaccination with pIRES I or pIRES II Induces gD-specific T Cell Responses and Resistance to HSV-1 Challenges

The trivalent nature of the immune responses elicited in the mice immunized with pIRES I or pIRES II was confirmed after determination of the HSV gD-specific T cell responses. Two weeks after the last immunization dose, BALB/c mice were monitored for the induction of gD-specific CD4$^+$ and CD8$^+$ T cell responses using ELISPOT assays. As shown in **Figure 4A**, mice vaccinated with pIRES I or pIRES II developed similar antigen-specific cellular responses, as measured by the number of IFN-γ-secreting cells after stimulation with purified gD, compared with mice immunized with the empty vector. Analysis of the activated antigen-specific T cell populations by flow cytometry indicated that both pIRES I and pIRES II activated the CD4$^+$ and CD8$^+$ T cells. Nonetheless, the mice immunized with pIRES II activated a higher proportion of gD-specific IFN-γ producing CD8$^+$ T cells

Figure 3. Induction of *in vivo* E7- and p24-specific cytolytic CD8^{+} T cell responses in immunized mice. (A–B) *In vivo* antigen-specific CD8^{+} T cell-dependent cytotoxic responses in the vaccinated mice was measured two weeks after the last immunization dose. Spleen cells from BALB/c or C57BL/6 mice were labeled with CFSE and pulsed with synthetic peptides representing the immunodominant MHC-I-restricted epitopes of p24 (A) or E7 (B). Data shown in A and B represent the compilation of two independent experiments, encompassing four and five mice per group (n = 9) with results based on the response of each animal. (C) The protective immunity elicited in BALB/c mice immunized with pIRES I or pIRES II was measured after challenge with a recombinant vaccinia virus expressing the HIV-1 protein Gag. Female BALB/c mice were challenged with 2×10^6 P.F.U. of rVV-Gag, and 5 days later, the level of viable vaccinia virus in the ovaries was determined after titration in Vero cells. Data shown in C represent the compilation of two independent experiments carried out with pooled samples from five mice per group. *p<0.05. (D) Prophylactic and (E) therapeutic anti-tumor immunity in C57BL/6 mice immunized with pIRES I or PIRES II. The prophylactic anti-tumor effects were determined in five vaccinated female mice after the s.c. transplantation of 7.5×10^4 TC-1 cells two weeks after the last vaccination. The therapeutic anti-tumor effects induced by the vaccines were determined after transplantation of 7.5×10^4 TC-1 cells one day before the administration of the first vaccine dose. Data shown in D and E represent the compilation of two independent experiments, with five mice per group. The survival curves D and E raised *p* values of 0.0001 and 0.0006, respectively, in the Logrank test for trend. pIRES is the empty vector used as immunization control.
doi:10.1371/journal.pone.0071322.g003

(CD8+/CD4+ ratio of 1.2) than mice immunized with pIRES I (CD8+/CD4+ ratio of 0.64) (**Figure 4B**). pIRES I- and pIRES II-vaccinated mice were also partially protected against lethal challenge with HSV-1. Sixty percent of the BALB/c mice immunized with pIRES II survived the lethal challenge carried out with HSV-1 EK strains, while a lower level of protection (30%) was achieved in mice immunized with pIRES I (**Figure 4C**). In conclusion, these results also confirm that the tested DNA vaccines induce T cell-dependent immune responses and protective immunity against HSV.

Materials and Methods

Ethics Statement

All animal handling and immunization procedures were approved by the institutional ethics committee for animal experimentation and care "*CEUA ICB-USP – Comissão de Ética no Uso de Animais do Instituto de Ciências Biomédicas da Universidade de São Paulo*" (protocol number: 12/05/2010-007-82-2) and followed standard rules approved by the Brazilian College of Animal Experimentation (COBEA).

Mice, Cell Lines and Antibodies

C57BL/6 and BALB/c mice (6–8 weeks old) were provided by the animal facility of the Department of Parasitology at the University of São Paulo. The TC-1 tumor cell lineage [34], which is derived from C57BL/6 mouse lung epithelial cells transformed with the v-Ha-ras oncogene and HPV-16 E6 and E7, was kindly provided by Dr T. C. Wu at John Hopkins University. TC-1 and COS-7 (ATCC® CRL-1651™) cells were cultured in Dulbeccos modified Eagle's 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 after trypsinization, washed twice, and suspended in serum-free media at the appropriate concentrations for injection. Rabbit anti-gD, mouse anti-p24 and mouse anti-E7 polyclonal antibodies were generated in our laboratory by
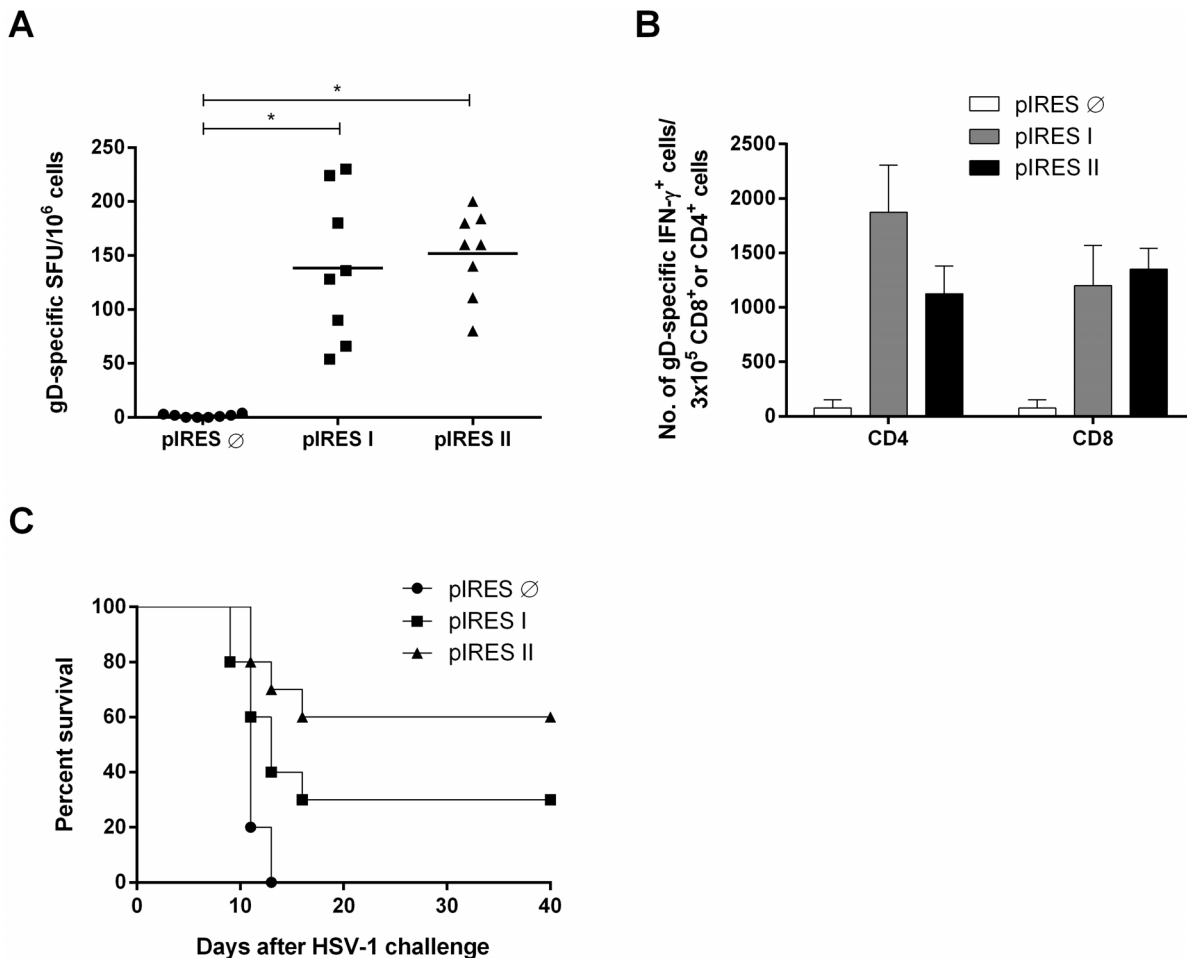

Figure 4. Induction of gD-specific functional T cell responses in mice immunized with pIRES I or pIRES II. (A) Detection of gD-specific IFN-γ-secreting cells in vaccinated BALB/c mice was performed two weeks after the last immunization dose. Spleen cells from individual mice (n = 8) were cultured in the presence of full-length recombinant purified gD for 72 h. The frequencies of the gD-specific IFN-γ-secreting cells were measured by ELISPOT. (B) Detection of gD-specific IFN-γ-secreting CD8+ and CD4+ T cells in those mice described in (A) with spleen cells previously incubated for 72 h with the recombinant gD analyzed by flow cytometry. Data shown in A and B represent the compilation of two independent experiments, with four mice per group (n = 8). *p<0.05. (C) Protective anti-HSV immunity elicited in mice immunized with pIRES I or pIRES II. Vaccinated male BALB/c mice were challenged intranasal with the HSV-1 strain EK (5×10^4 P.F.U./mouse) two weeks after last vaccine dose and mice survival was monitored for 40 days. Data shown in C represent the compilation of two independent experiments, with five mice per group (n = 10). $p = 0.0009$ (Logrank test for trend). pIRES is the empty vector used as immunization control.
doi:10.1371/journal.pone.0071322.g004

subcutaneous injection of four doses of the specific recombinant proteins in combination with alum.

Cloning of gDp24 and gDE7 in the pIRES Vector

The gDE7 and gDp24 gene fusions were obtained by PCR amplification using the plasmids pgDE7 [27] and pgDp24 as templates, which contain the coding sequences of HPV-16 E7 and HIV-1 clade B p24, respectively, each fused to the coding sequence of HSV-1 gD. The inserts were amplified using the primers gDFwXbaI (5′ TAG <u>TCT AGA</u> ATG GGG GGG GCT GCC GCC AGG 3′, *XbaI* restriction site underlined) and gDFwNheI (5′ TAG <u>GCT AGC</u> ATG GGG GGG GCT GCC GCC AGG 3′, *NheI* restriction site underlined) for subcloning upstream of the IRES or the primers gDRvNotI (5′ TAG <u>GCG GCC GCG</u> CAC CCA TTA AGG GGG GGT ATC 3′, *NotI* restriction site underlined) and gDRvEcoRI (5′ TAG <u>GAA TTC</u> GCA CCC ATT AAG GGG GGG TAT C 3′, *EcoRI* restriction site underlined) for subcloning downstream of the IRES. The amplicons were cloned into the pGEM™ cloning vector and then double digested with *NheI/EcoRI* or *XbaI/NotI* (Invitrogen, Carlsbad, CA). The resulting fragments were subcloned into the pIRES™ vector (Clontech, Mountain View, CA). The plasmid containing the gDp24 gene fusion upstream of the IRES and gDE7 downstream of the IRES was named pIRES I. The plasmid containing the gDE7 gene fusion upstream of the IRES and gDp24 downstream of the IRES was named pIRES II. All plasmid constructs were confirmed by automatic DNA sequencing. The final vectors were propagated in *Escherichia coli* DH5-α in LB medium supplemented with ampicillin (100 μg/mL) and purified using a Qiagen Plasmid Mega Kit (Qiagen, Hilden, Germany).

Cell Transfection and Immunofluorescence Analysis

COS-7 cells were cultivated in 12-well culture plates at an initial concentration of 2×10^5 cells/well in DMEM supplemented with 10% FBS. On the following day, the cells were transfected using Lipofectamine™ 2000 (Invitrogen) according to the manufacturers instructions. After 36 h, the cells were harvested with PBS containing 2 mM EDTA, pelleted and incubated with blocking solution (1% bovine serum albumin in PBS) for 20 min at room temperature. The cells were washed twice with PBS and then incubated for 45 min with a 1:100 dilution of a rabbit specific anti-gD serum and a mouse specific anti-E7 serum or the same anti-gD serum combined with a mouse specific anti-p24 serum. The cells were washed twice with PBS and then incubated for 45 min with a 1:200 dilution of FITC-labeled goat anti-mouse IgG (Sigma) or a 1:200 dilution of Texas Red-labeled goat anti-rabbit IgG (Sigma). The cells were washed twice with PBS, stained with a 1:1,000 dilution of DAPI (1 mg/mL) and visualized for immunofluorescence using an inverted microscope (Axiovert model S100, Zeiss) equipped with a digital camera (Hamamatsu model C5810, Sanyo Denki).

DNA Vaccination and *in vivo* Protection Experiments

BALB/c or C57BL/6 mice were intramuscularly (i.m.) immunized with 3 doses of the DNA vaccines at weekly intervals, with 100 μg DNA/dose in PBS in a final volume of 100 μL. Tumor growth prevention was determined after subcutaneous transplantation of 7.5×10^4 TC-1 cells/mouse in the right flank two weeks after the last immunization. Therapeutic anti-tumor effects were measured after transplantation of the TC-1 cells and administration of ththe first vaccine dose one day later. Tumor growth was monitored by visual inspection and palpation twice per week over an observation period of 60 days. The prophylactic anti-HSV effects in the vaccinated BALB/c mice were determined using the HSV-1 EK strain (kindly provided by Dr. Erna Geessien Kroon of the Universidade Federal de Minas Gerais), which was diluted in PBS, and 10-μL aliquots (containing 5×10^4 P.F.U./dose) were delivered in the mouse nostrils 15 days after the last immunization dose. The challenged mice were monitored for survival for 40 days.

To evaluate the prophylactic anti-HIV immune response induced by the vaccines, mice were challenged with recombinant vaccinia virus expressing the HIV-1 protein Gag (rVV-Gag), which was supplied by the NIH (vaccinia strain vABT-141). Challenge experiments were performed by i.p. inoculating female BALB/c mice with 2×10^6 P.F.U. of rVV-Gag in 100 μL of saline two weeks after the last immunization. The mice were euthanized, and their ovaries were harvested and macerated with a syringe plunger in 1 mL DMEM. The suspensions were submitted to 2-fold serial dilution, 100 μL of each dilution was incubated with Vero cells for 1 h, and the medium was replaced. After 72 h, the cells were stained with a solution containing 20% alcohol and 0.2% of violet crystal, and subsequently inspected for the viral plaques.

Determination of CD8+/IFN-γ⁺ T Cell Response by Intracellular Cytokine Staining (ICS) and ELISPOT Assays

Spleen cells were harvested two weeks after the last immunization dose, suspended in RPMI 1640 supplemented with 5% SFB and treated with ACK lysing buffer for 5 min on ice to eliminate the red blood cells. For intracellular IFN-γ staining, spleen cells (3×10^6 cells/well) were cultured at 37°C in 96-well round-bottom microtiter plates (BD Biosciences) in 200 μL of RPMI 1640 medium supplemented with penicillin (100 U/mL), streptomycin (100 μg/mL), 2-ME (50 mM), L-glutamine (2 mM), sodium pyruvate (1 mM), 10% heat-inactivated FBS and brefeldin A (GolgiPlug, BD Biosciences) at a final concentration of 10 μg/mL. In vitro stimulation was achieved using the MHC-I-restricted (H-2K^b) E7-specific peptide (E7$_{49-57}$; RAHYNIVTF) or the MHC-I-restricted (H-2K^d) p24-specific peptide (gag$_{197-205}$; AMQMLK-ETI) at a concentration of 3 μg/mL for 6 h or recombinant gD at a concentration of 10 μg/mL for 72 h (brefeldin A was added in the last 6 h of culture). The cells were incubated for 30 min at 4°C with 100 μL of a 1:100 dilution of FITC-conjugated mAb anti-mouse CD8a and Pe-Cy5-conjugated mAb anti-mouse CD4 (BD Biosciences), washed 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. After washing, the cells were incubated for 30 min at 4°C with a 1:100 dilution of PE-labeled mAb anti-mouse IFN-γ (BD Biosciences). The cells were examined with a FACSCalibur™ cytometer (BD Biosciences), and the data were analyzed using FlowJo software (Tree Star). ELISPOT assays were carried out after the incubation of splenocytes with the same stimuli used in the ICS assay but for 24 hours for peptide stimulation and 72 hours for protein stimulation. Spleen cells (5×10^5/well) were seeded for in vitro stimulation in nitrocellulose-bottom plates that were previously coated with anti-IFN-γ. IFN-γ secretion was detected after staining with a biotinylated anti-IFN-γ antibody overnight at 4°C and treatment with the AEC Substrate Set (BD Biosciences) according to manufacturer's instructions.

In vivo Antigen-specific Cytotoxicity Assays

Naïve mouse splenocytes were stained with 0.5 μM or 5 μM of carboxy fluorescein diacetate succinimidyl ester (CFSE) (Invitrogen). The cells labeled with 5 μM CFSE were pulsed with 2.5 μg/mL of the E7-specific peptide (RAHYNIVTF) or the p24-specific (AMQMLKETI) peptide for 40 min at 37°C. Equal amounts of both cell populations (2×10^7 cells) were intravenously (i.v.)

inoculated into C57BL/6 or BALB/c mice, for determination of anti-E7 or anti-p24 responses, respectively, that were previously submitted to the immunization regimens two weeks after the last immunization dose. One day later, the spleens of the inoculated mice were harvested and monitored by flow cytometry. The percentage of target cell killing by specific cytotoxic CD8^{+} T lymphocytes (CTL) was determined as previously described [35].

Statistical Analyses

All data, which are expressed as means ± SD, are representative of at least five mice per group, and all experiments were repeated twice. One-way ANOVA followed by Bonferroni's post-test was employed whenever individual data points were compared. The survival and tumor development curves were submitted to log-rank tests. Differences with $p \leq 0.05$ were considered to be statistically significant.

Discussion

The three most relevant viral pathogens associated with sexually transmitted diseases, HIV, HSV and HPV, share common features, such as mucosal transmission, the ability to establish chronic infections, and the requirement of antigen-specific cytotoxic CD8^{+} T cell activation for the efficient control of intracellular virus replication. So far the search for vaccines against these pathogens has emphasized strategies involving the induction of antibodies that are able to neutralize the viruses. Indeed, such approaches have been successful, as two preventive and lucrative anti-HPV vaccines have been generated and are currently being used in several countries [3,4]. Nonetheless, there is a strong belief that the immunological control of HIV and HSV as well as the tumors induced by HPV, either prophylactic or therapeutically, will require the implementation of alternative methodological tools. In the present study, we propose a trivalent vaccine approach in which bicistronic DNA vaccines simultaneously encoding antigens of HIV, HSV and HPV activate antigen-specific and functionally active CD8^{+} T cell responses under experimental conditions. Moreover, our study demonstrates that the tested vaccine formulations confer protective immunity to all three antigens from HIV, HPV and HSV viruses, as observed in mice challenged with an HSV-1 strain or a recombinant vaccinia virus encoding the HIV-1 protein Gag and in mice implanted with tumor cells expressing the HPV-16 oncoproteins. Altogether, our results indicate that the development of multivalent vaccines inducing protective immunity against HIV, HSV and HPV are feasible and further support the relevance of cytotoxic T cell responses for the protective immunity to these pathogens.

Our proposed vaccine strategy is based on two methodological approaches that induce protective immunity. First, the use of bicistronic DNA vectors that allow for the simultaneous expression of two chimeric proteins (HIV-1 p24 and HPV-16 E7 that are both genetically fused to the HSV-1 envelope protein) gD in the same transfected cells permits a more balanced and efficient induction of CD8^{+} T cell responses in vaccinated subjects. Second, chimeric proteins in which both the HIV-1 protein p24 and the HPV-16 oncoprotein E7 are fused to the HSV-1 protein gD at its C-terminus are targeted to the surface of the transfected cells. In particular, this second feature results in the induction of gD-specific immunomodulatory pathways and the subsequent enhanced activation of CD8^{+} T cell responses to the encoded antigens and, therefore, to the protective immunity generated in the vaccinated animals [15,32,36]. The rationale behind the use of DNA vaccine vectors is well established and includes the potential to activate cellular immune responses without the use of exogenous

adjuvants, the lack of interference from previously established immunity, an excellent safety record and a rather simple and low-cost production pipeline [37]. Characteristically, DNA vectors, like most eukaryotic genes, are monocistronic, with a single structural gene under the control of a promoter. The discovery of IRES sequences revealed the possibility of generating polycistronic DNA vectors, leading to the simultaneous activation of immune responses to different antigens without the need to transfect cells several plasmids [23,24,38,39]. Our results confirmed that both of our DNA vectors constructed with this IRES technology promoted the synthesis of the target antigens in the same transfected cell. In addition, immunization of mice with pIRES I or pIRES II elicited antigen-specific CD8^{+} T cell responses and in vivo cytotoxic responses. These results may reflect the differential expression of the antigens encoded by the two cistrons, with higher expression for the proximal genes compared with the distal cistron. Similar effects have been previously reported by other groups [24,25,40], and most likely reflect premature transcript termination at the IRES sequence, which reduces the level of full-length mRNA and, consequently, the amount of the protein encoded by the second cistron. Induction of a more balanced immune response to the target antigens could, thus, be achieved after the concomitant administration of both pIRES I and II vectors. Nonetheless, the present results indicate that the bicistronic expression technology still needs further improvement in order to be successfully used at in vivo situations, particularly, as vaccines.

The efficient activation of T cell responses involves the correct processing and surface presentation of epitopes by APCs and the correct signaling between APCs and effector T cells, which is mediated by surface-exposed receptors, by means of different co-stimulatory and co-inhibitory pathways [41,42]. The HSV-1 protein gD encoded by our DNA vaccines plays dual roles in the induction of protective anti-viral responses. First, it acts as a highly conserved protective antigen for both the HSV-1 and HSV-2 strains, and second, it exerts strong and complex immunomodulatory effects, enhancing the activation of the CD8^{+} T cell response to its genetically fused antigens [29,43]. The entry of both HSV-1 and HSV-2 into the host cell is mediated, at least in part, by the specific interaction between gD and HVEM, a member of the TNFR family [44]. In addition, the binding of gD to HVEM blocks the co-inhibitory pathway by competing with BTLA and CD160 [43]. In contrast, the binding of gD does not interfere with the co-stimulatory functions of HVEM, which involve other surface proteins, such as LIGHT and lymphotoxin-α, and lead to the stimulation of the transcription factors NF-kβ and AP-1 and the subsequent activation and differentiation of effector T cells [45,46]. Further experimental evidence has demonstrated that the binding of gD to HVEM activates the NF-kβ-dependent signaling pathway irrespective of additional HVEM-binding factors [30,31]. Previous results, based both on DNA vaccines and adenovirus vectors, have demonstrated that the fusion of heterologous sequences at the C-terminus of gD target the protein to the outer face of the cytoplasmic membrane where the binding to HVEM occurs, leading to the enhanced activation of CD8^{+} T cell responses to the heterologous antigen [13,15,32,36]. Similar but less significant results have also been obtained with recombinant purified proteins [14], further suggesting that endogenous expression and membrane localization contributes to the gD-mediated CD8^{+} T cell adjuvant effects.

Induction of cytotoxic CD8^{+} T cell responses represents an important immunological correlate for vaccines aiming therapeutic activation of protective immunity, such as those involved with eradication of chronic infections and cancer [47,48]. Indeed, cytotoxic CD8^{+} T cells are involved in the resolution of infections

with HIV [49–51], HSV [52,53] and HPV-induced tumors [15,27,54]. The present study demonstrated that bicistronic DNA vaccines encoding gD fused antigens could enhance the induction of antigen-specific cytotoxic CD8$^+$ T cell responses and confer protective immunity to virus infection and therapeutic control of tumor growth. These findings also indicate that a trivalent vaccine formulation targeting HIV, HSV and HPV associated cancer is a feasible goal and represent an interesting approach to be tested in the near future.

Author Contributions

Conceived and designed the experiments: VCS MOD FAMOC AMV ECN RRA MAC GKL LCSF. Performed the experiments: VCS MOD FAMOC RRA GKL. Analyzed the data: VCS MOD FAMOC AMV ECN RRA MAC GKL LCSF. Contributed reagents/materials/analysis tools: VCS MOD FAMOC AMV ECN RRA MAC GKL LCSF. Wrote the paper: VCS MOD LCSF.

References

1. WHO (2012) Global report: UNAIDS report on the global AIDS epidemic 2012.

2. Nikolic DS, Piguet V (2010) Vaccines and Microbicides Preventing HIV-1, HSV-2, and HPV Mucosal Transmission. Journal of Investigative Dermatology 130: 352–361.

3. Paavonen J, Jenkins D, Bosch FX, Naud P, Salmeron J, et al. (2007) Efficacy of a prophylactic adjuvanted bivalent L1 virus-like-particle vaccine against infection with human papillomavirus types 16 and 18 in young women: an interim analysis of a phase III double-blind, randomised controlled trial. Lancet 369: 2161–2170.

4. Garland SM, Hernandez-Avila M, Wheeler CM, Perez G, Harper DM, et al. (2007) Quadrivalent vaccine against human papillomavirus to prevent anogenital diseases. New England Journal of Medicine 356: 1928–1943.

5. Lin K, Roosinovich E, Ma B, Hung C-F, Wu TC (2010) Therapeutic HPV DNA vaccines. Immunologic Research 47: 86–112.

6. Autran B, Carcelain G, Combadiere B, Debre P (2004) Therapeutic Vaccines for chronic infections. Science 305: 205–208.

7. Buchbinder SP, Mehrotra DV, Duerr A, Fitzgerald DW, Mogg R, et al. (2008) Efficacy assessment of a cell-mediated immunity HIV-1 vaccine (the Step Study): a double-blind, randomised, placebo-controlled, test-of-concept trial. Lancet 372: 1881–1893.

8. Belshe RB, Leone PA, Bernstein DI, Wald A, Levin MJ, et al. (2012) Efficacy Results of a Trial of a Herpes Simplex Vaccine. New England Journal of Medicine 366: 34–43.

9. Dropulic LK, Cohen JI (2012) The challenge of developing a herpes simplex virus 2 vaccine. Expert Review of Vaccines 11: 1429–1440.

10. Melief CJM, van der Burg SH (2008) Immunotherapy of established (pre)malignant disease by synthetic long peptide vaccines. Nature Reviews Cancer 8: 351–360.

11. van Steenwijk PJdV, Ramwadhdoebe TH, Lowik MJG, van der Minne CE, Berends-van der Meer DMA, et al. (2012) A placebo-controlled randomized HPV16 synthetic long-peptide vaccination study in women with high-grade cervical squamous intraepithelial lesions. Cancer Immunology Immunotherapy 61: 1485–1492.

12. Bagarazzi ML, Yan J, Morrow MP, Shen X, Parker RL, et al. (2012) Immunotherapy Against HPV16/18 Generates Potent T(H)1 and Cytotoxic Cellular Immune Responses. Science Translational Medicine 4.

13. Lasaro MO, Tatsis N, Hensley SE, Whitbeck JC, Lin S-W, et al. (2008) Targeting of antigen to the herpesvirus entry mediator augments primary adaptive immune responses. Nature Medicine 14: 205–212.

14. Porchia BFMM, Diniz MO, Cariri FAMO, Santana VC, Amorim JH, et al. (2011) 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. Molecular Pharmaceutics 8: 2320–2330.

15. Diniz MO, Lasaro MO, Ertl HC, Ferreira LCS (2010) Immune Responses and Therapeutic Antitumor Effects of an Experimental DNA Vaccine Encoding Human Papillomavirus Type 16 Oncoproteins Genetically Fused to Herpesvirus Glycoprotein D. Clinical and Vaccine Immunology 17: 1576–1583.

16. Donnelly JJ, Ulmer JB, Shiver JW, Liu MA (1997) DNA vaccines. Annual Review of Immunology 15: 617–648.

17. Moniz M, Ling M, Hung CF, Wu TC (2003) HPV DNA vaccines. Frontiers in Bioscience 8: D55–D68.

18. Almeida RR, Rosa DS, Ribeiro SP, Santana VC, Kallas EG, et al. (2012) Broad and Cross-Clade CD4(+) T-Cell Responses Elicited by a DNA Vaccine Encoding Highly Conserved and Promiscuous HIV-1 M-Group Consensus Peptides. Plos One 7.

19. Kong WP, Huang Y, Yang ZY, Chakrabarti BK, Moodie Z, et al. (2003) Immunogenicity of multiple gene and clade human immunodeficiency virus type 1 DNA Vaccines. Journal of Virology 77: 12764–12772.

20. Konishi E, Kosugi S, Imoto JI (2006) Dengue tetravalent DNA vaccine inducing neutralizing antibody and anamnestic responses to four serotypes in mice. Vaccine 24: 2200–2207.

21. Kwissa M, Unsinger J, Schirmbeck R, Hauser H, Reimann J (2000) Polyvalent DNA vaccines with bidirectional promoters. Journal of Molecular Medicine-Jmm 78: 495–506.

22. Mountford PS, Smith AG (1995) Internal ribosome entry sites and dicistronic RNAS in mammalian transgenesis. Trends in Genetics 11: 179–184.

23. Zhang H-Y, Sun S-H, Guo Y-J, Zhu W-J, Shi K, et al. (2008) Optimization strategy for plasmid DNAs containing multiple-epitopes of foot-and-mouth disease virus by cis-expression with IL-2. Vaccine 26: 769–777.

24. Liang R, Babiuk LA, Littel-van den Hurk SvD (2007) Compatibility of plasmids encoding bovine viral diarrhea virus type 1 and type 2 E2 in a single DNA vaccine formulation. Vaccine 25: 5994–6006.

25. Manoj S, Babiuk LA, Littel-van den Hurk SV (2003) Immunization with a dicistronic plasmid expressing a truncated form of bovine herpesvirus-1 glycoprotein D and the amino-terminal subunit of glycoprotein B results in reduced gB-specific immune responses. Virology 313: 296–307.

26. Shoji M, Yoshizaki S, Mizuguchi H, Okuda K, Shimada M (2012) Immunogenic Comparison of Chimeric Adenovirus 5/35 Vector Carrying Optimized Human Immunodeficiency Virus Clade C Genes and Various Promoters. Plos One 7.

27. Lasaro MO, Diniz MO, Reyes-Sandoval A, Ertl HC, Ferreira LCS (2005) 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 and Infection 7: 1541–1550.

28. Diniz MO, Ferreira LCS (2011) Enhanced anti-tumor effect of a gene gun-delivered DNA vaccine encoding the human papillomavirus type 16 oncoproteins genetically fused to the herpes simplex virus glycoprotein D. Brazilian Journal of Medical and Biological Research 44: 421–427.

29. Lasaro MO, Ertl HCJ (2009) Potentiating vaccine immunogenicity by manipulating the HVEM/BTLA pathway and other co-stimulatory and co-inhibitory signals of the immune system. Human Vaccines 5: 6–14.

30. Sciortino MT, Medici MA, Marino-Merlo F, Zaccaria D, Giuffre-Cuculletto M, et al. (2008) Involvement of gD/HVEM interaction in NF-kappa B-dependent inhibition of apoptosis by HSV-1 gD. Biochemical Pharmacology 76: 1522–1532.

31. Sciortino MT, Medici MA, Marino-Merlo F, Zaccaria D, Giuffre-Cuculletto M, et al. (2008) Involvement of HVEM receptor in activation of nuclear factor kappa B by herpes simplex virus 1 glycoprotein D. Cellular Microbiology 10: 2297–2311.

32. DiMenna L, Latimer B, Parzych E, Haut LH, Toepfer K, et al. (2010) Augmentation of Primary Influenza A Virus-Specific CD8(+) T Cell Responses in Aged Mice through Blockade of an Immunoinhibitory Pathway. Journal of Immunology 184: 5475–5484.

33. Watson RJ, Weis JH, Salstrom JS, Enquist LW (1982) Herpes-Simplex Virus Type-1 Glycoprotein-D Gene - Nucleotide-Sequence and Expression In Escherichia-Coli. Science 218: 381–384.

34. Lin KY, Guarnieri FG, Staveley-O'carroll KF, Levitsky HI, August JT, et al. (1996) Treatment of established tumors with a novel vaccine that enhances major histocompatibility class II presentation of tumor antigen. Cancer Research 56: 21–26.

35. Barber DL, Wherry EJ, Ahmed R (2003) Cutting edge: Rapid in vivo killing by memory CD8 T cells. Journal of Immunology 171: 27–31.

36. Lasaro MO, Sazanovich M, Giles-Davis W, Mrass P, Bunte RM, et al. (2011) Active Immunotherapy Combined With Blockade of a Coinhibitory Pathway Achieves Regression of Large Tumor Masses in Cancer-prone Mice. Molecular Therapy 19: 1727–1736.

37. MacGregor RR, Boyer JD, Ugen KE, Lacy KE, Gluckman SJ, et al. (1998) First human trial of a DNA-based vaccine for treatment of human immunodeficiency virus type 1 infection: Safety and host response. Journal of Infectious Diseases 178: 92–100.

38. Benvenisti L, Rogel A, Kuznetzova L, Bujanover S, Becker Y, et al. (2001) Gene gun-mediate DNA vaccination against foot-and-mouth disease virus. Vaccine 19: 3885–3895.

39. Shkreta L, Talbot BG, Diarra MS, Lacasse P (2004) Immune responses to a DNA/protein vaccination strategy against Staphylococcus aureus induced mastitis in dairy cows. Vaccine 23: 114–126.

40. Mizuguchi H, Xu ZL, Ishii-Watabe A, Uchida E, Hayakawa T (2000) IRES-dependent second gene expression is significantly lower than cap-dependent first gene expression in a bicistronic vector. Molecular Therapy 1: 376–382.

41. Wang SD, Chen LP (2004) Co-signaling molecules of the B7-CD28 family in positive and negative regulation of T lymphocyte responses. Microbes and Infection 6: 759–766.

42. Croft M (2003) Co-stimulatory members of the TNFR family: Keys to effective T-cell immunity? Nature Reviews Immunology 3: 609–620.

43. Cai G, Freeman GJ (2009) The CD160, BTLA, LIGHT/HVEM pathway: a bidirectional switch regulating T-cell activation. Immunological Reviews 229: 244–258.

44. Spear PG, Longnecker R (2003) Herpesvirus entry: an update. Journal of Virology 77: 10179–10185.

45. Marsters SA, Ayres TM, Skubatch M, Gray CL, Rothe M, et al. (1997) Herpesvirus entry mediator, a member of the tumor necrosis factor receptor (TNFR) family, interacts with members of the TNFR-associated factor family and activates the transcription factors NF-kappa B and AP-1. Journal of Biological Chemistry 272: 14029–14032.

46. Granger SW, Rickert S (2003) LIGHT-HVEM signaling and the regulation of T cell-mediated immunity. Cytokine & Growth Factor Reviews 14: 289–296.

47. Appay V, Douek DC, Price DA (2008) CD8(+) T cell efficacy in vaccination and disease. Nature Medicine 14: 623–628.

48. Seder RA, Hill AVS (2000) Vaccines against intracellular infections requiring cellular immunity. Nature 406: 793–798.

49. Cossarizza A, Bertoncelli L, Nemes E, Lugli E, Pinti M, et al. (2012) T Cell Activation but Not Polyfunctionality after Primary HIV Infection Predicts Control of Viral Load and Length of the Time without Therapy. Plos One 7.

50. Buckheit RW III, Salgado M, Silciano RF, Blankson JN (2012) Inhibitory Potential of Subpopulations of CD8(+) T Cells in HIV-1-Infected Elite Suppressors. Journal of Virology 86: 13679–13688.

51. Saez-Cirion A, Lacabaratz C, Lambotte O, Versmisse P, Urrutia A, et al. (2007) HIV controllers exhibit potent CD8 T cell capacity to suppress HIV infection ex vivo and peculiar cytotoxic T lymphocyte activation phenotype. Proceedings of the National Academy of Sciences of the United States of America 104: 6776–6781.

52. Orr MT, Mathis MA, Lagunoff M, Sacks JA, Wilson CB (2007) CD8 T cell control of HSV reactivation from latency is abrogated by viral inhibition of MHC class I. Cell Host & Microbe 2: 172–180.

53. Khanna KM, Bonneau RH, Kinchington PR, Hendricks RL (2003) Herpes simplex virus-specific memory CD8(+) T cells are selectively activated and retained in latently infected sensory ganglia. Immunity 18: 593–603.

54. Cheng WF, Hung CF, Chai CY, Hsu KF, He LM, et al. (2001) Tumor-specific immunity and antiangiogenesis generated by a DNA vaccine encoding calreticulin linked to a tumor antigen. Journal of Clinical Investigation 108: 669–678.