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Prime–boost therapeutic vaccination in mice with DNA/DNA or DNA/Fowlpox virus recombinants expressing the Human Papilloma Virus type 16 E6 and E7 mutated proteins fused to the coat protein of Potato virus X

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Highlights

- FP-E6$_{F47R}$CP and FP-E7$_{GGG}$CP correctly express transgenes in different cell lines
- After the genetic immunization, tumor volume was lower than in the control mice
- Genetic immunization delayed tumor appearance compared to control mice
- After DNA/FP vaccination, better results were obtained by E6/E7 single immunogens

Abstract

The therapeutic antitumor potency of a prime–boost vaccination strategy was explored, based on the mutated, nontransforming forms of the E6 (E6$_{F47R}$) and E7 (E7$_{GGG}$) oncogenes of Human Papilloma Virus type 16 (HPV16), fused to the Potato virus X (PVX) coat protein (CP) sequence. Previous data showed that CP fusion improves the immunogenicity of tumor-associated antigens and may thus increase their efficacy. After verifying the correct expression of E6$_{F47R}$CP and E7$_{GGG}$CP inserted into DNA and Fowlpox virus recombinants by Western blotting and immunofluorescence, their combined use was evaluated for therapy in a pre-clinical mouse model of HPV16-related tumorigenicity. Immunization protocols were applied using homologous (DNA/DNA) or heterologous (DNA/Fowlpox) prime–boost vaccine regimens. The humoral

immune responses were determined by ELISA, and the therapeutic efficacy evaluated by the delay in tumor appearance and reduced tumor volume after inoculation of syngeneic TC-1* tumor cells. Homologous DNA/DNA genetic vaccines were able to better delay tumor appearance and inhibit tumor growth when DNAE6$_{F47R}$CP and DNAE7$_{GGG}$CP were administered in combination. However, the heterologous DNA/Fowlpox vaccination strategy was able to delay tumor appearance in a higher number of animals when E6$_{F47R}$CP and in particular E7$_{GGG}$CP were administered alone.

Keywords: HPV therapeutic vaccines; Fowlpox virus recombinants; prime/boost immunizations; Potato virus X coat protein; E6 and E7 modified oncoproteins

1. Introduction

The Human Papilloma Virus (HPV) is responsible for more than 550,000 new cases of human cancers each year, and the high-risk HPV16 and HPV18 genotypes represent the primary etiologic agents of cervical tumors (Roden and Wu, 2003). HPV16 and HPV18 are also implicated in the majority of the ano-genital tumors of both sexes (Zandberg et al., 2013) and in a subset of head and neck cancers (Worsham et al., 2012)**.** After infection, the E6 and E7 oncoproteins of HPV16 bind and interact with specific cell substrates to generate an immune environment that cannot control tumorigenicity and contributes to cancer initiation and progression (Münger et al., 1989; Song et al., 2000). In particular, E6 and E7 deregulate fundamental cellular events, such as the cell cycle, apoptosis, DNA repair, cell senescence and differentiation (Duensing and Münger, 2004; Hudson et al., 1990)**.** Although effective prophylactic vaccines have been introduced to prevent high-risk HPV infection and the development of cervical tumors, the therapeutic efforts to annihilate HPV-associated diseases still remain a critical issue (Knoff et al., 2014)**.** On this basis, both DNA expression plasmids and viral-vectored immunogens have many inherent features that make them promising vaccine candidates.

Genetic vaccines can elicit good immune responses, and they are relatively stable, inexpensive, susceptible to genetic manipulations, and safe also for immunocompromised individuals, as they do not contain live or inactivated pathogens (Gurunathan et al., 2000). Recently, a bivalent therapeutic vaccine (VGX-3100) showed efficacy against HPV16 and HPV18, thus offering a possible option for the prevention of cervical cancer by eliciting HPV-specific immunity. Also, in a phase III efficacy trial, the novel 9-valent HPV prophylactic vaccine (9vHPV) demonstrated 96.7% efficacy against vulvar and vaginal intraepithelial neoplasia grade 2 or 3, caused by HPV31, HPV33, HPV45, HPV52, or HPV58 (Livermore, 1995). However, preclinical studies have already demonstrated that DNA-based vaccines encoding the E6 or the E7 proteins are less immunogenic and effective than those in which these oncogenes are fused to carriers (Knoff et al., 2014; Rosales and Rosales, 2014**).** Carriers can improve antigen presentation and tumor eradication, and they include calreticulin (Peng et al., 2004), *Mycobacterium tuberculosis* heat-shock protein-70, Herpes simplex virus type 1 (HSV-1) VP22 protein and lysosome-associated membrane protein-1 (Peng et al., 2005), *Escherichia coli* β-glucuronidase (Poláková et al., 2010), tetanus toxin fragment C (Oosterhuis et al., 2011), and Simian virus 40 J-domain and enhancer (Almajhdi et al., 2014). Moreover, as a general drawback, DNA vaccines show low immunogenicity and efficacy in nonhuman and human primates (Pereira et al., 2014)**,** and it can be important to know whether immunogenicity can be enhanced by boosting with poxvirus-based recombinants also when DNA priming is performed only once.

Vaccinia virus-based recombinants have already been used successfully to enhance antigen immunogenicity in prime–boost protocols, where they were primed by DNA-based E6/E7 genetic vaccines (Chen et al., 2000; Mackova et al., 2006). However, to limit the induction of neutralizing antibodies against this replicative vector, alternative safe non-replicative poxvirus vectors are under evaluation, which can also overcome the only partially abortive replication in mammals of the Modified Vaccinia virus Ankara (Blanchard et al., 1998; Drexler et al., 1998)**.** With this aim, a non-pathogenic Fowlpox virus (FP) poultry vaccine strain has already been engineered as a recombinant vector against different human infectious diseases, and it has also been used in pre-clinical studies for HPV (Radaelli et al., 2012)**.** Avian poxviruses are replication-restricted to avian species (Taylor and Paoletti, 1988) and they do not cross-react immunologically with mammalian poxviruses. They can elicit a complete immune response in vaccinated

hosts (Zanotto et al., 2010), and can escape pre-existing immunity in smallpox-experienced humans. They thus represent safe immunogens that are permissive for entry and transgene expression in most mammalian cells (Bissa et al., 2013; Pacchioni et al., 2013). In particular, FP has recently received the approval for clinical use in humans (www.ClinicalTrials.gov Identifier: NCT00083603), and FP-based recombinants combined with DNA in prime–boost immunization regimens have already shown greater protection when mice were primed with DNA followed by a FP boost (Radaelli et al., 2007). Although immune responses to HPV-related tumors include antibodies and TNF-α (Frazer et al., 2004; Malejczyk et al., 1992), tumor cells can be mainly eliminated by antigen-specific cytotoxic T-lymphocytes (Frazer et al., 2011; Li et al., 2010) and heterologous prime–boost regimens are among the most potent strategies to induce a cell-mediated immunity (Anderson and Schneider, 2007). The use of plasmid DNA combined with FP recombinant vaccines to deliver tumor-specific antigens might thus overcome the low immunogenicity of E6 and E7.

In our previous study, DNA and FP recombinants were generated that expressed a mutant HPV16 E6 antigen ($E6_{F47R}$) defective for the degradation of the p53 cell substrate. These recombinants were evaluated in preventive and therapeutic immunization protocols using a pre-clinical mouse model and following prime–boost vaccination regimens (Bissa et al., 2015). The results established a preliminary indication for the use of DNA and FP-based constructs as safe and effective immunogens, but also the necessity to improve their immunogenicity to enhance their antitumor efficacy.

Antigen fusion strategies increase vaccine stimulation of T cells that have escaped tolerance mechanisms and might have a particular potential for immunotherapy (Massa et al., 2008). DNA vaccines that encoded the HPV16 E5, E6 and E7 proteins genetically fused to HSV-1 glycoprotein D have already shown promising results in the control of cervical cancer in the preclinical mouse model (Diniz et al., 2010). However, neutralizing antibodies that target the glycoprotein D region required for the vaccine efficacy were reported in HSV-1-seropositive humans (Whitbeck et al., 1999). On this basis, the new class of plant-virus-derived sequences of the Potato virus X (PVX) coat protein (CP) can be a suitable carrier for antigen display. CP is recognized as a good immunogen for humans, and it can aggregate into structures that can increase $CD4^+$ T-cell–mediated protection (Savelyeva et al., 2001; Savelyeva et al., 2003). PVX and PVX-CP have already been fused to different viral antigens for transient expression in plant and epitope-

presentation systems (Cerovska et al., 2013; Franconi et al., 2002; Marusic et al., 2001) to develop more immunogenic vaccines. $E7_{GGG}$CP DNA induced a significantly higher cell-mediated response compared to vaccines based on $E7_{GGG}$ (Massa et al., 2008)**,** showed greater reduction of TC-1* tumor cell growth, and enhanced survival in a therapeutic setting.

In the present study, genetic and FP recombinants that encode the mutated non-oncogenic $E6_{F47R}$ and $E7_{GGG}$ proteins were fused to the 5'-terminus coding sequence of PVX-CP. The new recombinants were administered in homologous (DNA/DNA) and heterologous (DNA/FP) prime–boost vaccination regimens to improve the safety and efficacy of therapeutic vaccines against HPV16-related tumors.

2. Materials and methods

2.1. Cell lines

Specific-pathogen-free primary chick embryo fibroblasts (CEFs) were grown in Dulbecco's modified Eagle's medium supplemented with 5% heat-inactivated calf serum (Gibco Life Technologies, Grand Island, NY, USA), 5% tryptose phosphate broth (Difco Laboratories, Detroit, MI, USA), and 100 U/ml penicillin and 100 mg/ml streptomycin. CaSki cells, which contain multiple copies of the integrated HPV16 DNA genome, green monkey kidney (Vero) cells, and normal human lung fibroblasts (MRC-5 cells) were grown in Dulbecco's modified Eagle's medium supplemented with 10% calf serum, and 100 U/ml penicillin and 100 mg/ml streptomycin. TC-1 star (TC-1*), a clone of TC-1 cells (Venuti et al., 2009)**,** generally used for challenge to test putative prophylactic or therapeutic vaccines in the preclinical mouse model, was selected for its 100% tumorigenicity in challenged mice. TC-1 cells are derived from primary epithelial cells of C57BL/6 mice, and cotransformed with the HPV16 E6 and E7 and by the activated c-Ha-ras oncogene (Lin et al., 1996)**.** Before inoculation, the TC-1* cells were trypsinized and harvested, washed twice, and resuspended in Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (PBS-) at 5×10^5 cells/ml.

2.2. Viruses

The FP wild type (FPwt) and the FP-$E6_{F47R}$CP, FP-$E7_{GGG}$CP and FP-gagpol recombinants were amplified in CEFs, sucrose gradient purified, and titered before use. The FP recombinant containing the irrelevant gag

and pol genes of HIV-1 (FP-gagpol) (Radaelli et al., 2003) was used as a negative control for animal immunization.

2.3. DNA-E6$_{F47R}$CP and DNA-E7$_{GGG}$CP expression plasmids

DNA-E6$_{F47R}$CP was constructed by fusion of the E6$_{F47R}$ gene in frame to the fourth codon of PVX-CP. The genes were assembled using splicing by overlap extension-polymerase chain reactions. For E6$_{F47R}$CP fusion, the E6$_{F47R}$ gene was amplified using the forward E6BH3 (5'-GGC-CGG-ATC-CAA-GCT-TAT-CAT-GCA-CCA-AAA-GAG-AAC-TGC-AAT-G-3') and reverse CPE6 (5'-GGC-CTG-TGT-TGT-GCT-AGC-TGG-CAG-CTG-GGT-TTC-TCT-TAC-G-3') primers to incorporate the upstream part of PVX-CP and the downstream nucleotides of the E6 gene (5'-GGC-CTG-TGT-TGT-GCT-AGC-TGG-CAG-CTG-GGT-TTC-TCT-TAC-G-3'). For CP amplification, the forward E6CP (5'-CGT-AGA-GAA-ACC-CAG-CTG-CCA-GCT-AGC-ACA-ACA-CAG-GCC-3') and reverse CPNP (5'-TTC-TCG-ACT-TGC-GGC-CGC-CTG-CAG-TTA-TGG-TGG-TGG-TAG-AGT-GAC-3') primers were used to incorporate the E6 gene downstream nucleotides. The assembled genes were cloned into the pcDNA3.1(+) mammalian expression vector after digestion with BamHI and NotI restriction enzymes, to obtain the DNA-E6$_{F47R}$CP construct.

All of the plasmids were propagated in *E. coli* XL1-Blue, prepared using EndoFree plasmid Maxi kits (Qiagen, Hilden, Germany), diluted in PBS-, and used for cell transfection or animal immunization. The preparation of the DNA-E7$_{GGG}$CP was as already described by Massa *et al*. (Massa et al., 2008).

2.4. Construction of the FP-E6$_{F47R}$CP and FP-E7$_{GGG}$CP recombinants

FP-E6$_{F47R}$CP and FP-E7$_{GGG}$CP that expressed the E6$_{F47R}$CP and E7$_{GGG}$CP proteins were obtained by *in-vitro* homologous recombination (Pacchioni et al., 2010)**.** Briefly, the genetically mutated E6$_{F47R}$CP and E7$_{GGG}$CP genes of HPV16 were obtained from the DNAE6$_{F47R}$CP and DNAE7$_{GGG}$CP plasmids, and were inserted into the pFP$_{MCS}$ vector downstream of the VVH6 Vaccinia virus early/late promoter. The pFP$_{MCS}$ vector contained the 3-β-hydroxysteroid dehydrogenase 5-delta 4 isomerase gene which was interrupted by a multiple cloning site (Rosel et al., 1986). The DNA sequences that encoded the E6$_{F47R}$CP and E7$_{GGG}$CP regions were verified using the forward V62 (5′-GGT-TCT-TGA-GGG-TTG-TGT 3′) and reverse V350 (5′-

ATA-CAT-CGA-CCG-GTC-CAC 3′) primers for the $E6_{F47R}CP$ gene, and the forward V62 and reverse V101 (5′-CGA-AGC-TTT-TAT-GGT-TTC-TGA-GAA-CAG 3′) primers for the $E7_{GGG}CP$ gene. The amplification was carried out as described previously (Pozzi et al., 2009)**.** The recombination plasmids were purified, and the $E6_{F47R}CP$ and $E7_{GGG}CP$ genes were sequenced to exclude any possible mutations, and designated as pFP-$E6_{F47R}CP$ (9403 bp) and pFP-$E7_{GGG}CP$ (9210 bp). The viral recombinants were obtained by *in-vitro* recombination in primary CEFs, as described previously (Radaelli and De Giuli Morghen, 1994), using FPwt and pFP-$E6_{F47R}CP$ or pFP-$E7_{GGG}CP$ (Zanotto et al., 2011). The recombinant clones were identified by autoradiography after plaque hybridization with the [^{32}P]-labeled $E6_{F47R}CP$ and $E7_{GGG}CP$ probes, and then subjected to multiple cycles of plaque purification. One clone for each construct was selected for its correct and high expression of the $E6_{F47R}CP$ and $E7_{GGG}CP$ genes, as determined by RT-PCR, Western blotting and immunofluorescence. The FP-$E6_{F47R}CP$ and FP-$E7_{GGG}CP$ recombinants were amplified in CEFs, purified on a discontinuous sucrose gradient, titered, and used for mice immunization.

2.5. Western blotting

To determine whether the $E6_{F47R}CP$ and $E7_{GGG}CP$ proteins were correctly expressed, CEFs and Vero, NIH-3T3 and MRC-5 cells were infected with 10 PFU/cell FP-$E6_{F47R}CP$ or FP-$E7_{GGG}CP$ and examined by Western blotting, as described previously (Pozzi et al., 2009). For E6, the blotted nitrocellulose membranes were incubated overnight at 4 °C with a primary anti-E6 rabbit polyclonal antibody (AbE6P/MI) produced in our laboratory, with 1:4000 dilution for CEFs, and 1:1000 dilution for Vero, MRC-5 and NIH-3T3 cells. AbE6P/MI was generated by multiple inoculations of New Zealand White rabbits with the FP-E6 recombinant followed by E6 protein boosts. Before using, the serum was immmunoadsorbed overnight either with CEFs, Vero, MRC-5 or NIH-3T3 cells infected with FPwt. For E7, different monoclonal antibodies were used for the different cell lines: the 8C9 mouse anti-E7 monoclonal antibody (Zymed Lab., Invitrogen Corp., San Diego, CA) was used at 1:100-dilution for CEFs and Vero and MRC-5 cells. For the detection of $E7_{GGG}CP$ in the NIH-3T3 cell line, the ED17 mouse monoclonal antibody (AbE7/SC; Santa Cruz biotechnology, Santa Cruz, CA, USA) was used at 1:100 dilution. After a 1-h incubation with the secondary horseradish-peroxidase-conjugated anti-rabbit or anti-mouse goat antiserum (DakoCytomation,

Carpinteria, CA, USA) at 1:2000 dilution, and 2-h washes, the proteins were revealed using the ECL system (GE Healthcare, Buckinghamshire, UK). Cells infected with FPwt were used as the negative control, whereas CaSki cells, and the E6 and E7 proteins as positive controls.

2.6. Immunofluorescence

To detect the expression and intracellular localization of the $E6_{F47R}CP$ and $E7_{GGG}CP$ proteins, immunofluorescence was also carried out on CEFs and Vero, MRC-5, and NIH-3T3 cells infected with 10 PFU/cell, as described previously (Zanotto *et al*., 2011). The cells were seeded at a density of 3-5 $\times 10^5$/35-mm^2 dish on sterile glass coverslips, and grown overnight before infection with 10 PFU/cell of either FP-$E6_{F47R}CP$ or FP-$E7_{GGG}CP$, at 37 °C for 1 h. After an overnight incubation at 37 °C, the cells were washed twice with PBS-, fixed with 2% paraformaldehyde (Polysciences, Inc., Warrington, PA, USA) in PBS- for 10 min, and then with 100% cold acetone for 5 min at 4 °C. For both E6 and E7, the rabbit polyclonal antibodies AbE6P/MI and AbE7P/MI were used for the different cell lines, followed by FITC-goat anti-rabbit antiserum (DakoCytomation). AbE7P/MI was produced as already described for E6. Serum was used at 1:800 dilution in CEFs, and at 1:300 dilution in Vero and MRC-5 cells for detection of $E6_{F47R}CP$, and at 1:20 dilution for all of the cell lines for detection of $E7_{GGG}CP$. The samples were viewed under a fluorescence microscope (Zeiss Axioskop).

2.7. Production in bacteria of the E6 and E7 oncoproteins in their native form

To obtain the HPV16 His_6-E6 and His_6-E7, the pQE30 expression plasmid (Qiagen) was engineered to contain the E6 or E7 genes (referred to as pQE30-E6/His or pQE30-E7/His, respectively), and inserted into the M15 strain of *E. coli* (Qiagen). The proteins were prepared under non-denaturing conditions. Briefly, after 16 h growth, the culture was 100-fold diluted in fresh Luria–Bertani broth with 25 μg/ml kanamycin and 100 μg/ml ampicillin, then incubated at 37 °C until 0.6-0.7 OD_{600} was reached. Expression of the His_6-E6 and His_6-E7 proteins was induced with 1 mM isopropyl-β-D-thiogalactopyranoside. The cells were maintained at 28 °C or 30 °C (for His_6-E6 and His_6-E7, respectively) for 16 h, and harvested by centrifugation at 4000× *g* for 20 min at 4 °C. The pellet was resuspended in lysis buffer (50 mM NaH_2PO_4,

200 mM NaCl, with 100 μM dithiothreitol, pH 7.5, or 20 mM imidazole pH 8.0) that contained an EDTA-free anti-protease cocktail (Roche, Basel, Switzerland), as recommended by the manufacturer. After adding 1 mg/ml lysozyme and 1% Triton X-100, the cells were incubated for 1 h at 4 °C and sonicated on ice at a 10-Hz output (1 min or 10 s for His_6-E6 or His_6-E7 respectively, thrice) with an ultrasonic disintegrator (Soniprep 150; MSE, London, UK). Clarification was by centrifugation at 15000× *g* for 45 min at 4 °C, and the supernatant was incubated for 16 h or 1 h (for His_6-E6 or His_6-E7, respectively) with 1 ml Ni-nitrilotriacetic acid agarose resin (Ni-NTA, Qiagen), previously equilibrated in lysis buffer. After washing several times with 50 mM NaH_2PO_4, 200 mM NaCl, and 70 mM imidazole, to a final 0.01 OD_{280}, the protein was eluted using 50 mM NaH_2PO_4, 200 mM NaCl, 300 mM imidazole, and run on 15% SDS-PAGE. All of the buffers used for the His_6-E6 protein purification were at pH 7.5 and contained 100 μM dithiothreitol. The elution fractions enriched in the recombinant His_6-E6 and His_6-E7 proteins were pooled, quantified, and stored until use at 4 °C and -20 °C, respectively. The purified E6 (pE6) and E7 (pE7) proteins were dialyzed in PBS- containing either 0.02% betaine and 100 μM dithiothreitol for pE6, or 0.1 mM $ZnCl_2$ for pE7. Both proteins were used for the enzyme-linked immunosorbent assays (ELISA).

2.8. Animal immunization

Six-week-old female C57BL/6 mice were purchased from Charles River (Como, Italy) and maintained under specific pathogen-free conditions at the Experimental Animal Department of the Regina Elena Cancer Institute (Rome, Italy).

Two therapeutic prime–boost protocols were performed: the first using only genetic immunogens, and the second using combined genetic/FP recombinant vaccines. In the first protocol (Fig. 1A), four groups of five mice were inoculated by subcutaneous injection (s.c.) in the flank with 5×10^4 TC-1* tumor cells/mouse, and vaccinated after 3 days and 10 days. Immunization was performed i.m. with 100 μg/mouse of each recombinant DNA (protocol G1: DNA-$E6_{F47R}$CP; protocol G2: DNA-$E7_{GGG}$CP; protocol G3: DNA-$E6_{F47R}$CP + DNA-$E7_{GGG}$CP). The mice for protocol G4 were mock vaccinated and used as controls. In the second protocol (Fig. 1B), after TC-1* tumor cell inoculation, the mice of G1, G2, and G3 (five mice/group) were primed once after 3 days with the same plasmids used in the genetic vaccination, and boosted s.c. at 1

week and 2 weeks after the priming with 10^7 PFU/mouse of each FP recombinant (protocol G1: FP-$E6_{F47R}$CP; protocol G2: FP-$E7_{GGG}$CP; protocol G3: FP-$E6_{F47R}$CP + FP-$E7_{GGG}$CP). A group of mice was vaccinated with the DNA and FP recombinants that contained the unrelated gag and pol transgenes as a negative control (protocol G4: DNA-gagpol, 100 μg/mouse, i.m.; FP-gagpol, 2×10^7 PFU/mouse, s.c.). Tumor growth was monitored by visual inspection and palpation three times a week. Tumor size was measured with calipers, and the volume estimated using the formula ($width^2 \times length \times 0.5$). The mice were euthanized for ethical reasons when tumors reached a volume of about 4 cm^3.

The protocols were approved by the Ethical Committee and were developed according to European Guidelines no. 86/609/CEE and 116/92 for the protection of laboratory experimental animals and laboratory animal care (Ministry of Health, Department of Veterinary Public Health, Nutrition and Food Security, Protocol 17/2006).

2.9. ELISA

The ELISA was essentially performed as described previously (Bissa *et al*., 2013), using the purified native HPV16 E6 or E7 proteins, and CaSki cells or MRC-5 cells (negative control) as plate-bound antigens. Briefly, 96-well maxisorp microtiter plates (Nunc, Naperville, IL, USA) were coated with pE6 or pE7 (300 ng/well) in PBS-, or with CaSki or MRC-5 lysates (10^5 cells/well) in 0.05 M carbonate–bicarbonate buffer, pH 9.6, and incubated overnight at 4 °C. The CaSki and MRC-5 cells were disrupted by freeze–thawing three times. The sera of the animals were examined separately and used at 1:50 and 1:100 dilutions, and the binding was revealed using horseradish-peroxidase-conjugated goat anti-mouse sera at 1:2000 dilution (DakoCytomation) and tetramethylbenzidine substrate (Sigma-Aldrich Italia, Milan, Italy). The absorbance for each well was measured at 450 nm with a microplate reader 550 (Bio-Rad Laboratories, Hercules, CA, USA).

2.10. Statistical analyses

Statistical analyses were performed using one-way ANOVA parametric tests and Bonferroni analysis of variance, using the GraphPad Prism 5 software. Statistical significance was set as $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***).

3. Results

3.1. FP-E6$_{F47R}$CP and FP-E7$_{GGG}$CP recombinants express the modified E6 and E7 transgenes fused to CP

Fusion of the mutated E6$_{F47R}$ (506 bp) and E7$_{GGG}$ (326 bp) genes to the CP gene (732 bp) generated the expected bands of 1179 bp and 999 bp that corresponded to the E6$_{F47R}$CP and E7$_{GGG}$CP genes, respectively (data not shown). The genetically mutated E6$_{F47R}$CP and E7$_{GGG}$CP genes inserted into the FP vectors by *in-vitro* homologous recombination expressed the mutated fusion proteins in all of the infected CEF and Vero, MRC-5 and NIH-3T3 cell lysates, as demonstrated by Western blotting. These showed as a 43-kDa protein band for E6$_{F47R}$CP (Fig. 2A, I, lanes 2) and a 40-kDa protein band for E7$_{GGG}$CP (Fig. 2, II, lanes 2). No specific bands were seen when these cells were infected with FPwt (Fig. 2A, I and II, lanes 1). The non-mutated His$_6$-E6 and His$_6$-E7 proteins produced by an engineered bacterial vector were used as positive controls (Fig. 2A, I and II, lanes 3).

3.2. E6 and E7 expressed by FP-E6$_{F47R}$CP and FP-E7$_{GGG}$CP are mainly in the cytoplasm

FP-E6$_{F47R}$CP (Fig. 2B; 1b-4b) and FP-E7$_{GGG}$CP (Fig. 2B; 1c-4c) recombinants were also used to infect CEFs and Vero, MRC-5 and NIH-3T3 cells, to determine by immunofluorescence the cellular localization of the E6 and E7 proteins. The E6 and E7 staining was mainly cytoplasmic and diffuse in CEFs (Fig. 2B; 1b and 1c), but granular in the mammalian cell lines (Fig. 2B; 2b-4b, 2c-4c). The immunofluorescence was similar to that observed in the same cells infected with FP-E6$_{F47R}$ and FP-E7$_{GGG}$ (data not shown). The intensities of the fluorescence signals were also greater for E6 than for E7. The FPwt-infected cells were always negative, as expected (Fig. 2B; 1a-4a).

3.3. Tumor appearance and development were more delayed when DNA-E6$_{F47R}$CP and DNA-E7$_{GGG}$CP were administered in combination

After the genetic immunization (Fig. 3; see Fig. 1A for protocol groups), when DNA-E6$_{F47R}$CP was compared to DNA-E7$_{GGG}$CP, and both the immunogens were used either alone or in combination, 100% of the G4 mock-immunized mice developed tumors at day 14 post-inoculation (p.i.). Conversely, tumor appearance was significantly delayed to day 19 p.i. ($p < 0.05$) in 100% of the mice of the G1 and G2 groups. In the G3 group, tumor bearing mice, defined as the animals carrying a palpable tumor, were 80% at day 19 p.i. ($p < 0.05$) and reached 100% of the mice by day 24 p.i..

At 24 days p.i., the tumor volumes of the G1, G2, and G3 mice were smaller compared to those of the G4 mice (Table 1, $p < 0.001$; see Fig. 1A for protocol groups). Moreover, when DNA-E6$_{F47R}$CP and DNA-E7$_{GGG}$CP were administered in combination, the tumor volumes were also reduced, and were significantly smaller if compared to when DNA-E6$_{F47R}$CP was used alone (G3 *vs* G1, $p < 0.05$). After a week, the tumor volumes in all of the groups were further increased, and the mice were euthanized for ethical reasons.

3.4. Genetic/FP vaccines seem to give better results when the immunogens are used alone

After the genetic/FP recombinant immunizations (Fig. 4; see also Fig. 1B for protocol groups), when priming was performed with the single (E6$_{F47R}$CP or E7$_{GGG}$CP) or combined (E6$_{F47R}$CP + E7$_{GGG}$CP) DNA vaccines, followed by two boosts with the single or combined FP recombinants, tumors developed in 20% of G4 mice at day 12 p.i., reaching 40% by day 14, 80% by day 17 p.i. and 100% by day 28. Unexpectedly, a similar trend was observed in the animals of G3, that developed tumors at day 12 p.i. in 20% of the animals, reaching 100% by day 17 p.i.. Conversely, a delay in tumor appearance was observed in the G1 and G2 mice. In G1, the tumors first appeared in 60% of the mice at day 17 p.i., and in 100% of the animals by day 28 p.i. whereas in G2 tumor bearing animals were 20% at day 14 p.i., then reaching 60% at day 17 p.i., and 100% only by day 38 p.i.. At 33 days p.i., the tumor volumes were similar in G1 and G2, and much larger in G3 (data not shown).

3.5. The specific humoral response is almost undetectable

Mice immunized by the combined genetic/FP recombinants were tested for anti-E6-specific or anti-E7-specific humoral immunity. The antibody responses against the E6 and E7 proteins were determined by ELISA, with plates coated with either the purified native pE6 or pE7 proteins, or the CaSki cell lysates (Fig. 5). The MRC-5 cell lysates were used as a negative-control antigen. The mice sera were analyzed before injecting the TC-1* cells (T0), before the first and second boosts (T1, T2, respectively), and 1 week after the second boost (T3). The results are reported only after the third immunization (T3), as the means from each group of mice. By using the different plate-bound antigens, no clear differences in the humoral responses were found among the groups. Also when E7 or the CaSki cells were used, the observed response was very limited.

4. Discussion

Around 5% of all human cancers are related to HPV (Rosales, R. and Rosales, C., 2014), but no treatment is presently available to eliminate already established infections and prevent or delay disease recurrence. A therapeutic vaccine that targets the E6 and E7 oncoproteins might clear HPV malignancies by eliciting specific cytotoxic T-lymphocyte immune responses (Lin et al., 2010). This would also overcome the problem of the high numbers of HPV-infected subjects who might develop tumors of the cervix.

Vector design and antigen expression greatly influence the immunogenicity and efficacy of recombinant DNA and viral vaccines. To improve the antitumor immune responses, both DNA and recombinant viral vaccines have already been widely investigated, in attempts to combine efficacy with safety, immunogenicity, and low cost of production. Given the promising results obtained *in vivo* by the genetic $E7_{GGG}$CP vaccine (Massa et al., 2008)**,** a novel $E6_{F47R}$CP DNA construct was generated by fusing the attenuated HPV16 $E6_{F47R}$ gene with the PVX-CP gene. This plant virus CP represents a dominant pathogen-derived antigen and an innovative way to enhance the immunogenicity of the encoded product, as it increases the Th1 response (Massa et al., 2008; Savelyeva et al., 2001; Savelyeva et al., 2003) and reduces inappropriate immune responses and autoimmunity in therapeutic vaccination regimens.

In the present study, we demonstrated that: (i) FP-$E6_{F47R}$CP and FP-$E7_{GGG}$CP recombinants can correctly express the transgenes fused to CP in cell lines of different species; (ii) after the genetic

immunization, tumor appearance was delayed up to day 19 p.i. and tumor volume was lower at 24 days p.i. than in the control mice; (iii) after the heterologous DNA/FP vaccination strategy, better results were obtained with the single immunogens.

After generating the FP-E6$_{F47R}$CP and FP-E7$_{GGG}$CP recombinants, the transgene expression was evaluated in replication-permissive CEFs and non-permissive Vero, MRC-5 and NIH-3T3 cells by Western blotting and immunofluorescence. The analysis by Western blotting confirmed that all of the cell lines correctly expressed the recombinant proteins. In non-permissive cell lines, higher concentrations of antibody had to be used to reveal the E6$_{F47R}$CP protein, potentially because of low protein expression. In particular, in MRC-5 cells, the specific band was only visible when using larger amounts of cell lysate. The intensity of the bands was therefore not comparable because of the different conditions used in the different cell lines, including the different antibodies, dilutions, amounts of cell lysate, and lengths of film exposure. The presence of CP does not appear to modify the expression of E6 and E7, as there were similar immunofluorescence intensities when FP-E6$_{F47R}$CP-infected and FP-E7$_{GGG}$CP-infected cells were compared to cells infected with FP-E6$_{F47R}$ and FP-E7$_{GGG}$, which express E6 and E7 with no CP. The E6 and E7 cellular localization was mainly in the cytoplasm in all of the cell lines, as expected for foreign proteins synthesized by avipoxviral vectors. Interestingly, the E6 polyclonal antibody used for Western blotting also recognized the protein using immunofluorescence, thus demonstrating that it can detect both the native and denatured forms of the protein. Conversely, the E7 polyclonal antibody revealed the protein by Western blotting but not by immunofluorescence, thus showing its binding only to non-conformational epitopes.

After inoculation of the TC-1* tumor cells, the number of healthy animals was higher with administration of the DNA/DNA vaccines rather than the DNA/FP vaccines. A significant delay in tumor growth was observed in the mice following the DNA/DNA immunizations than could not be observed after the DNA/FP regimen. When the E6 and E7 DNA recombinants were administered in combination, tumor appearance was delayed, and when tumors developed their volumes were significantly smaller compared to the controls, which may be ascribed to the vaccine-induced cytotoxic T-lymphocyte response. Conversely, after the heterologous combined DNA/FP strategy, an improved protection against tumor development and growth were obtained with the single immunogens. In spite of the apparent contradictions in the results

obtained by the two protocols, this could be due to the double administration of DNA in the "genetic" immunization before boosting with FP recombinants.

As for the humoral response, poor antibody responses to certain antigens have already been described for the C57BL/6 mouse strain of mice (Mols-Vorstermans et al., 2013; Sellers et al., 2011), which showed an aspecific and elevated baseline response, that prevented the detection of specific antibodies. If this is expected using DNA vaccines, which appear to mainly elicit cell-mediated immunity, a response should be present after the boost with the FP recombinants.

The present study also confirms the data from our previous research where similar results were obtained after immunizing mice with only the DNA $E6_{F47R}$CP and $E7_{GGG}$CP vaccines by electroporation (Cordeiro et al., 2015). In contrast to that study, most of the mice remained tumor-free up to day 19 p.i.., instead of 13 days p.i.,

which can be ascribed to the different inoculation systems and the use of the different tumor cells for challenge.

The aim of this study was to verify the immunogenicity and efficacy of viral-vectored vaccines and the best association of the immunogens able to delay tumor appearance and growth, after fusing $E6_{F47R}$ and $E7_{GGG}$ to the CP of Potato virus X. It was also our aim to use the genetic and FP-based $E6_{F47R}$CP and $E7_{GGG}$CP recombinants in combination, which was not previously performed. In fact, it is already known that, although convenient because of their low cost and safety, DNA vaccines are poorly immunogenic in humans, and the use of FP-vectored vaccines as a boost could improve the efficacy. Although the combined genetic/FP strategy improved the outcome only when $E6_{F47R}$CP and $E7_{GGG}$CP were separately administered, the use of this regimen for $E6_{F47R}$CP and in particular for $E7_{GGG}$CP was able to delay tumor appearance in a higher number of animals than by the genetic immunization used alone.

Although the limited animal number used in this study might have prevented better statistical evaluations, the results of these immunization regimens suggest that the next strategy should include a repeated DNA administration by electroporation, which also reduces the amount of the vaccine antigen. It is also well known that the route of administration and vaccine dosage can affect immunogenicity (Bansal et al., 2008), as also shown by the improved immunological responses induced in mice by intranasal/oral immunization,

rather than the i.m. or s.c. routes (Lu et al., 2011). Given the advantages of mucosal vaccine delivered by aerosol, this way of immunization with recombinant avipoxvirus-based vaccines might improve immunogenicity and protection. Administration by the intratumoral route to deliver the transgenes directly into the tumor mass and fusion to the already tested adjuvants, such as the detoxified form of the saporin plant enzyme SAP-KQ (Massa et al., 2011)**,** might also ameliorate immunogenicity and efficacy.

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Conflict of interest

The authors declare that no conflict of interests exists.

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Figure legends

Fig. 1. Therapeutic immunization protocols.

(A) For the genetic vaccination regimen, four groups of five mice/group were injected s.c. with 5×10^4 TC-1* tumor cells/mouse and the immunogens (100 µg/mouse of each recombinant DNA) were administered i.m. 3 days and 10 days after TC-1* inoculation. The mice for protocol G4 were mock vaccinated and used as controls. (B) For the genetic/FP protocol, five mice/group were injected s.c. with 5×10^4 TC-1* cells, primed once i.m. after 3 days with recombinant DNA, and boosted s.c. (10^7 PFU/mouse of each FP recombinant) 1 week and 2 weeks after priming. The mice for protocol G4 were vaccinated with DNA and FP recombinants containing the unrelated gag and pol transgenes as a negative control. Arrows, times of TC-1* inoculation and vaccine administration; black triangles (T_0-T_3), bleeding times.

Fig. 2. WB analysis and cellular localization of $E6_{F47R}$CP and $E7_{GGG}$CP proteins expressed by the FP recombinants.

CEFs and Vero, MRC-5, and NIH-3T3 cells were infected at 10 PFU/cell with the FP-$E6_{F47R}$CP and FP-$E7_{GGG}$CP viral recombinants. The cell lysates were examined by Western blotting using the rabbit polyclonal AbE6P/MI antibody for the detection of E6 (2A, I, lanes 2), whereas different mouse monoclonal antibodies were used for the different cell lines for the detection of E7 (2A, II, lanes 2). The bands were revealed by horseradish-peroxidase-conjugated anti-rabbit or anti-mouse goat antiserum. The native E6 (2A, I, lane 3) and E7 (2A, II, lane 3) proteins were used as the positive controls (C^+). No specific proteins were seen when the cells were infected with FPwt (2A, I and II, lanes 1).

CEFs and Vero, MRC-5, and NIH-3T3 cells were infected with the FP-$E6_{F47R}$CP (2B, 1b-4b) and FP-$E7_{GGG}$CP (2B, 1c-4c) recombinants to determine the intracellular localization of the transgene products. Cytoplasmic staining was observed in all of the cell lines, as mainly diffuse in the CEFs (2B, 1b, 1c) and granular in the mammalian cells (2B, 2b-4b, 2c-4c). Rabbit polyclonal antibodies produced in our laboratory were used at different dilutions for the different cell lines: AbE6P/MI was used for E6, and AbE7P/MI for

E7, both followed by FITC-goat anti-rabbit antiserum. The immunofluorescence was greater for E6 than for E7, and was not seen when FPwt-infected cells were treated with anti-E6 or anti-E7 antibodies (2B, 1a-4a).

Fig. 3. Tumor bearing mice after the genetic therapeutic immunization.

C57BL/6 mice were injected s.c. with 5 $\times 10^4$ syngeneic TC-1* tumor cells and immunized 3 days and 10 days later (p.i.), according to the group protocols shown in Fig. 1. Priming was performed on day 3 p.i. with single DNA-E6$_{F47R}$CP (G1) or DNA-E7$_{GGG}$CP (G2) or combined DNA-E6$_{F47R}$CP + DNA-E7$_{GGG}$CP (G3) genetic vaccines, followed by a boost on day 10 p.i. with single (G1 or G2) or combined (G3) genetic vaccines. Mice of G4 were mock vaccinated and used as controls, and they all developed tumors at day 14 p.i.. Conversely, tumor appearance in the vaccinees was significantly delayed to day 19 p.i. (G1, G2, G3, $p < 0.05$). The animals carrying a palpable tumor were defined as tumor bearing animals.

Fig. 4. Tumor-bearing mice after DNA/FP recombinant immunizations.

C57BL/6 mice were inoculated with syngeneic TC-1* tumor cells and immunized 3 days, 10 days and 17 days later (p.i.), as shown in Fig. 1B. Priming was performed with single (E6$_{F47R}$CP or E7$_{GGG}$CP; G1 or G2) or combined (E6$_{F47R}$CP + E7$_{GGG}$CP; G3) genetic vaccines on day 3 p.i., followed by two boosts with single (G1 and G2) or combined (G3) FP recombinants on days 10 and 17 p.i.. Mice of G4, vaccinated with DNA and FP recombinants containing the gag and pol unrelated genes, developed tumors by day 12 p.i., reaching 100% by day 28 p.i.. Conversely, a delay was observed in G1, where tumors first appeared by day 17, reaching 100% of the animals by day 28 p.i., and in G2, where tumors first appeared by day 14, reaching 100% by day 38 p.i.. In the animals of G3, tumors developed following a trend similar to the control mice of the G4 group.

Fig. 5. Analysis of the E6-specific and E7-specific humoral responses in mice immunized with the genetic/FP recombinants.

Anti-E6 and anti-E7 antibody titers were determined by ELISA using either the native purified recombinant pE6 and pE7 proteins or CaSki cell lysates as plate-bound antigens. MRC-5 cell lysates were also plated as a

negative control. Data are reported as the means of the absorbance values using the sera of each group of mice after the third immunization. No clear differences in the humoral responses were found among the groups by using the different antigens and the results were very limited.

Fig 1

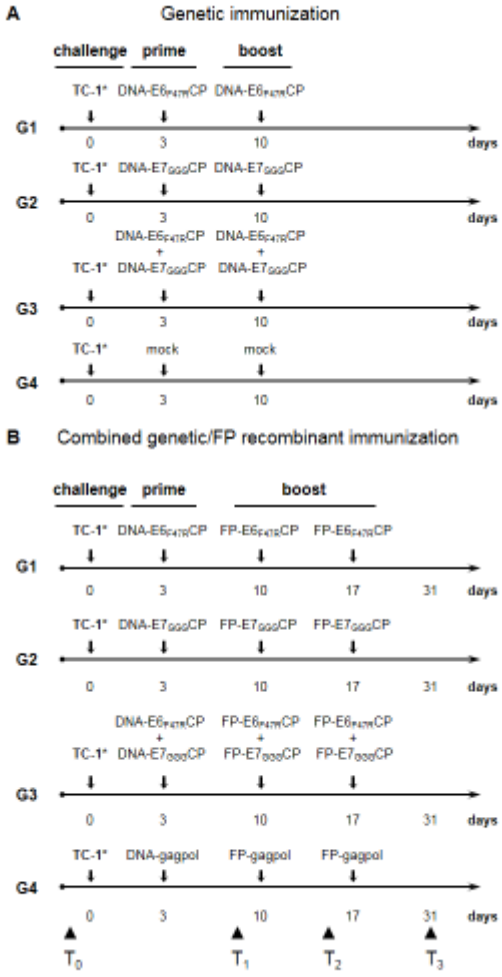

Fig2a

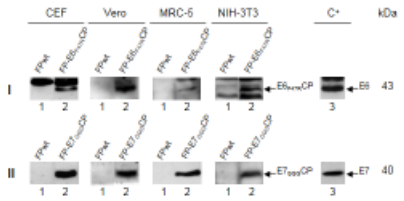

fig 2b

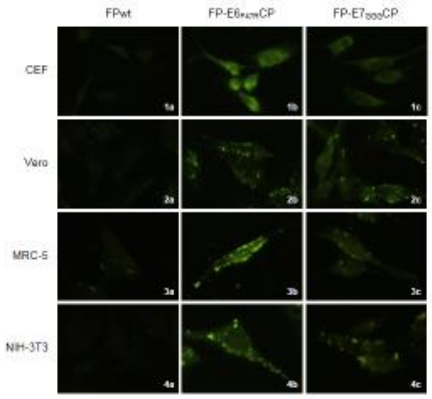

Fig. 3

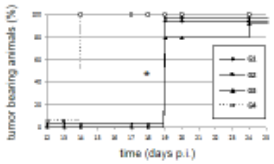

Fig 4

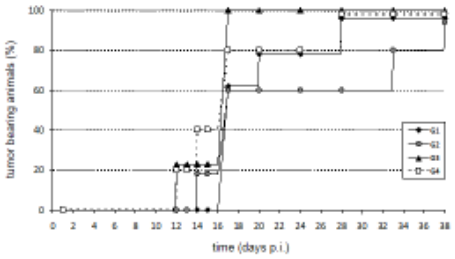

Fig 5

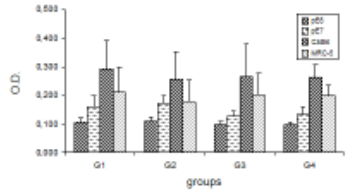

Table 1. Tumor volumes at 24 days p.i. in mice vaccinated with the DNA genetic recombinants

Vaccination protocol	Mean tumor volume (cm^3) 24 days p.i.
G1	1.71
G2	1.07
G3	0.53**
G4	3.19***

p.i., post inoculation
**, $p<0.05$,G3 *versus* G1
***, $p<0.001$, G1, G2, G3 *versus* G4