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Construction and evaluation of recombinant immunogens as therapeutic vaccines against HPV-related cancers

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ABSTRACT

Background

Considering the high number of new cases of cervical cancer each year caused by human papilloma viruses (HPVs), the development of an effective vaccine for the prevention and therapy of HPV-associated cancers, and in particular against the high-risk HPV16 genotype, remains a priority. Vaccines expressing the E6 and E7 proteins, which are detectable in all HPV-positive pre-cancerous and cancer cells, might support the treatment of HPV-related lesions and clear already established tumors.

Methods

In this study, DNA and fowlpox virus recombinants expressing the $E6_{F47R}$ and $E7_{GGG}$ mutated forms of the HPV16 E6 and E7 oncoproteins were generated, and their correct expression verified by RT-PCR, Western blotting and immunofluorescence. The immune responses were determined by ELISA and ELISPOT assays and the therapeutic efficacy was evaluated In mice, as a pre-clinical model of HPV-16 tumorigenicity, using heterologous (DNA/FP) or homologous (DNA/DNA and FP/FP) prime/ boost regimens after challenge with syngeneic TC-1* cells.

Results

The analyses of the different recombinants showed the correct expression of the inserted heterologous genes. After mice immunization, while specific anti-E6 and anti-E7 humoral responses were just detectable, specific T-cell responses were elicited. In the therapeutic

protocols, after the challenge and the subsequent immunizations, a delay in cancer appearance was shown, thus confirming the pivotal role of the T-cell response in the control of tumor growth also in the absence of E6- and E7-specific antibodies. These in-vivo experiments resulted in higher numbers of tumor-free mice after either the homologous or heterologous immunizations compared to the controls.

Conclusions

These data establish a preliminary indication for the prevention and treatment of HPV-related tumors by the use of DNA and avipox constructs as safe and effective immunogens administered by the prime/boost strategy. The combined use of the recombinants expressing both the $E6_{F47R}$ and $E7_{GGG}$ proteins should improve the antitumor efficacy and represent an important approach to control/clear HPV-associated cancers.

INTRODUCTION

Cervical cancer is the second most common malignancy among women worldwide [1,2] and approximately 500,000 new cases and 250,000 deaths are estimated each year [3]. There are more than 100 different genotypes of this virus, 15 of which are classified as “high-risk” and implicated in cancers of the cervix, penis, vulva, anus, vagina and oropharynx [4]. The casual factor of the disease is in fact the persistent infection of some strains of Human Papillomavirus (HPV), mainly spread by sexual-transmission.

HPV16 and HPV18 are the most diffused pathogenic HPVs and are associated with more than 70% of cervical cancers [2,5]. In particular, HPV16 is present in 50% of the tumors [1,2].

Infection restriction and prevention are difficult, since the transmission risk increases according to the sexual behavior, and, also by reducing the partner numbers and using the condom, a complete protection cannot be achieved because of the presence of infected cells also on the external part of the genitals [6].

The probability to be infected is further associated with others factors, such as smoking, number of sexual intercourses, age, pregnancy number, presence of other sexual transmitted diseases, and immunodeficiency (infection with Human Immunodeficiency Virus or pharmacological post-transplantation immune depression) [7].

The viral infection can also occur by vertical transmission from the mother to the child at the delivery [8], and can be facilitated in subjects that ignore their infectious status, since the currently performed preventive screenings on the female population are suggested but not mandatory.

The screening of sexually active women that are already twenty-one year old is currently performed by the Papanicolau test (Pap-test). This represents a fundamental way to fight the spreading of cervical cancer, a malignancy characterized by a slow-progression with an outbreak also after years from the infection event. The Pap-test analyzes the epithelial tissue samples, collected from the cervix, to the aim at detecting a typical perinuclear/cytoplasmic vacuolization (koilocytosis) that indicates the presence of HPV infection. (Fig. 1)

Unfortunately, this test is not predictive of HPV infection when the samples appear histologically normal, and further analyses must be performed to detect the presence of the viral genome. These additional analyses are generally prescribed only after a positive Pap-test result, so that, although these screenings are very diffused, the incidence of cervical cancer and the related deaths still remain high [8].

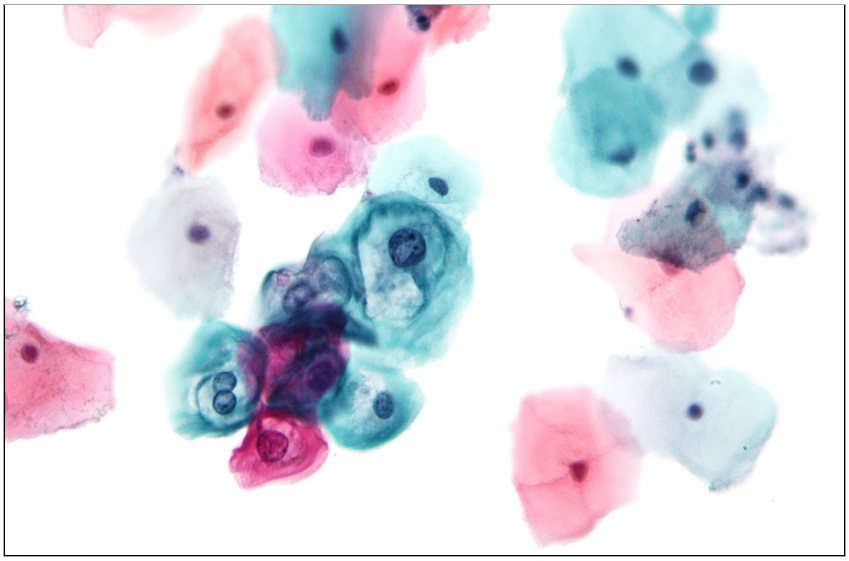

Fig. 1. Pap test. Micrograph showing a low grade squamous intraepithelial lesion (LSIL) after a Pap test. Abnormal cells have an enlarged nucleus, irregular chromatin and relatively abundant cytoplasm and may develop into cervical cancer.

The therapies currently pursued to treat the pre-cancerous lesions and the already developed cervical tumors involve surgery, as well as radio- and chemo-therapy. In general, these approaches are able to completely resolve the pathology only when the established Cervical Intraepithelial Neoplasia (CIN) belongs to the first or the second grade (CIN1 or CIN2), but they are insufficient in more severe cases of the third grade (CIN3) or carcinoma *in situ* [8]. During the last years, the scientific research was focused on the development of prophylactic and therapeutic vaccines able to induce both a humoral and cell-mediated immune response, that have been demonstrated to be involved in the spontaneous regression of the lesions that can occurs in some patients [6].

At the moment, two prophylactic vaccines against HPV are available, Gardasil® (Merck) and Cervarix® (Glaxo Smith-Kline). They consist of L1capsid protein of different HPV strains assembled into VLPs, that are able to induce a high antibody level, and thus, neutralize viral infection in the vaccinated subjects.

Both vaccines prevent 99% of infections mainly through the induction of neutralizing antibodies against the specific HPV types employed in the vaccine development. However, the induced humoral response is not sufficient to control the already established infection; thus, they are effective only in subjects vaccinated before the HPV transmission [9].

Despite the current preventive vaccines, cervical cancers are still on the rise with over 80% occurring in the poorest countries. This opens up the urgent need of developing therapeutic strategies targeting HPV-infected (pre)malignant cervical lesions through the elicitation of a cell-mediated immunity by an HPV-specific immunotherapy. The development of therapeutic vaccines for the treatment of cervical cancers is based on the possibility to induce a cell-mediated immune response targeting the early-expressed HPV proteins to prevent the development of new lesions and clear the already established ones.

The Human Papillomavirus (HPV)

The Human Papillomavirus (HPV) belongs to the family of *papillomaviridae* and to the genus of *papillomavirus*. HPVs are double-stranded DNA viruses and they present a specific tropism for the squamous epithelial cells of the skin or of the anogenital and oropharyngeal mucosae.

The viral particle (Fig. 2) has a diameter of 55 nm and an icosahedral capsid without envelope, which consists of the L1 and L2 structural proteins, containing a circular DNA of 7,904 bp [4].

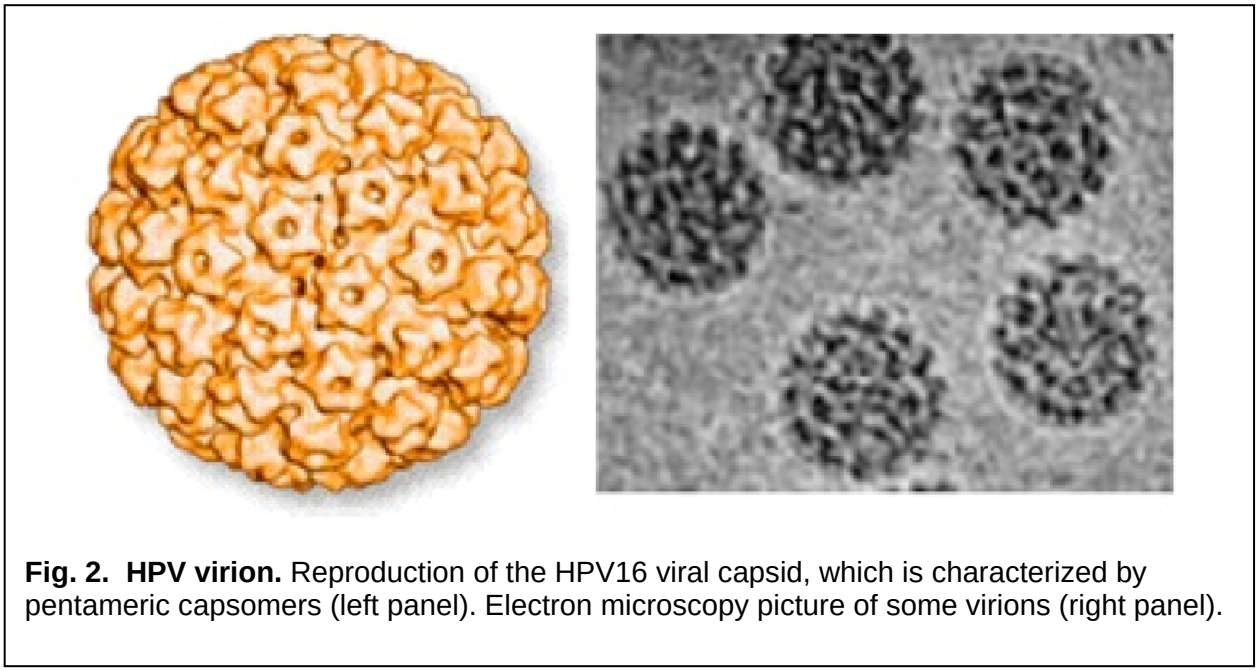

Fig. 2. HPV virion. Reproduction of the HPV16 viral capsid, which is characterized by pentameric capsomers (left panel). Electron microscopy picture of some virions (right panel).

The genome (Fig. 3) is expressed differently during the viral replication stages, and soon after infection, it appears as the episomal form, non-integrated in the genetic material of the host cell. HPV genome consists of 8 open reading frames, 6 early genes (E1, E2, E4, E5, E6, and E7), and 2 late genes (L1, and L2), the product of which vary from plain capsid proteins to immortalization tools, and a Long Control Region (LCR). Early genes are expressed in the basal, suprabasal, and intermediate cells of the cervix, during the initial stages of the viral cycle, and code for regulatory proteins, whereas the late genes, coding for the capsid proteins, are activated in the apical strata only during the final steps of the replication. Between the early and late regions, a regulatory Long Control Region (LCR) is present, which has a dimension of 1 kb and contains the origin-of-replication sequence.

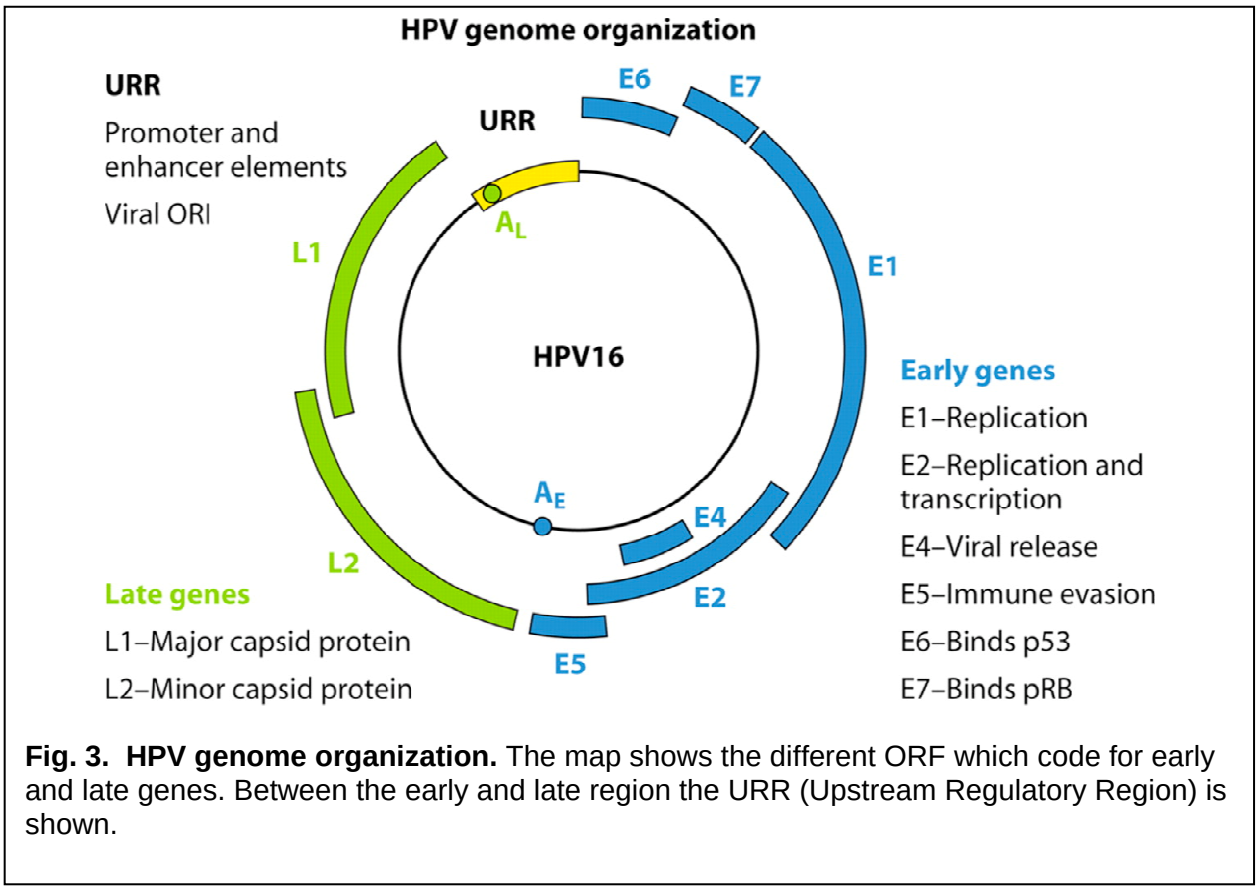

Fig. 3. HPV genome organization. The map shows the different ORF which code for early and late genes. Between the early and late region the URR (Upstream Regulatory Region) is shown.

Each early protein has specific functions: E1 prepares the viral genome to be replicated by the host replication machinery; E2 maintains the episomal form of the viral genome and organizes its transcription; E5 protein modifies (when expressed) the function of growth factor receptors; E6 and E7 are the major oncogenic tools in the viral genome. E4 full potential is yet to be clarified, but its expression appears throughout the epithelium, and it seems to promote viral replication and disrupt the cytoskeleton in order to facilitate the virions escape out of the differentiated cells [10].

The HPV tropism is species-specific and limited to the epithelial cells, where the viral replication takes place at the nuclear level where the expression of the different viral genes is strictly related at the differentiation stages of the cells [6].

In particular, the mature virion production occurs only in the spinous and granular most differentiated layers of the epithelium [11]. To allow a productive infection, the virus has to infect the cells of the basal epithelium, which are the only ones able to duplicate and provide the cells for the external layers [12].

After the penetration and the uncoating, that take place in the endosome, the DNA is released in the cytoplasm and translocated to the nucleus by the viral L2 protein. At this site, the transcription and duplication of the viral genome can begin, but they are still related to the differentiation state of the epithelium. These events occur in cells arrested in the G0 phase of the cell cycle, and depend on the replication machinery of the host cell, which is maintained active by the E6 and E7 early protein activity to allow the replication of the viral genome [13]. Moreover, the progression of the cell cycle is necessary during the first stages of the infection and for the viral gene expression [14]. Later during the viral replication, the L1 and L2 proteins spontaneously assemble into capsids, and the mature viral particles are released by desquamation of the epithelium [12] (Fig. 4).

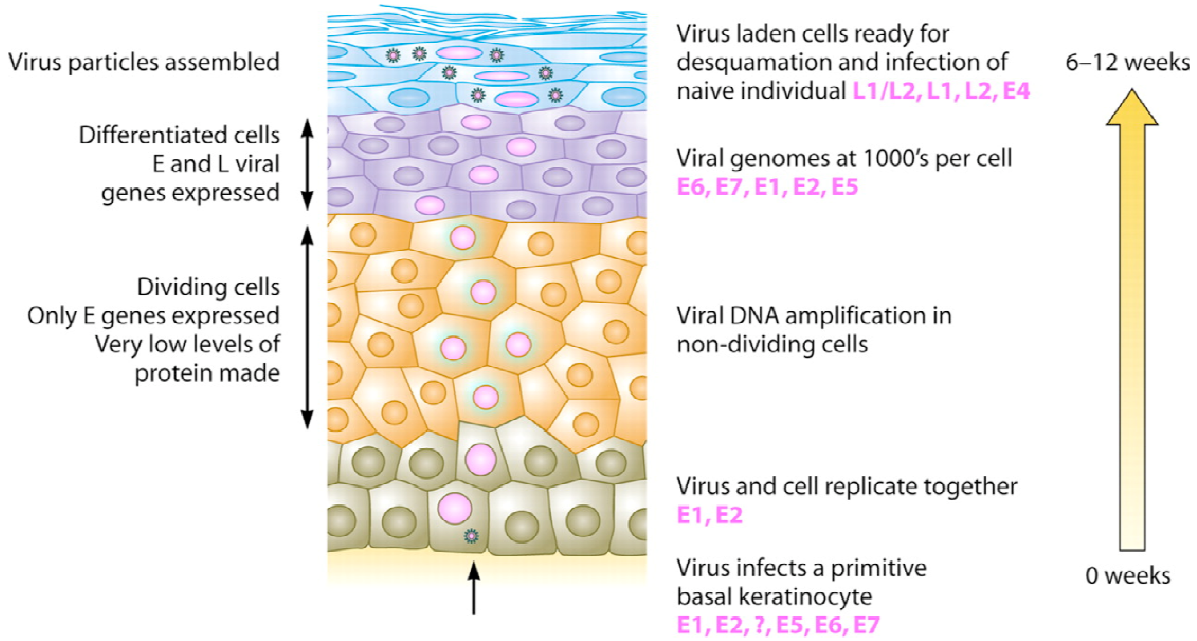

Fig. 4. HPV infection stages. Papillomaviruses are species-specific and tissue-specific viruses. HPV infects and replicates in the fully differentiating squamous epithelium only. The virus infectious cycle involves both temporal and spatial separation of viral protein expression. The virus first infects a keratinocyte in the basal layer of the epithelium. For the oncogenic viruses, viral gene expression is very tightly controlled during the proliferative phase. When the host cell stops dividing and begins to differentiate into a mature keratinocyte, this provides a signal to the virus to activate all of its genes to increase the viral genome copy number. In the case of incipient malignancy, the control of E6 and E7 expression is lost, and gene expression in the cells becomes deregulated. In the top layers of the epithelium, all of the viral genes, including those encoding the L1 and L2 proteins, are expressed, and many thousands of viral genomes are encapsidated.

HPV viruses have a distinct capability to evade the human immune system, which is achieved through three basic viral properties. First, immune cells cannot approach the virus easily since HPV has no viraemic phase. The initial infection is sited at the basal epithelium, whereas Langerhans cells are abundant only in the apical layers of the mucosa. Second, HPV does not elicit a major damage to the host cells, such as lysis, thus minimizing the inflammation and the subsequent signaling, and allowing the virus to replicate "silently" [15,16]. The third evasion mechanism is the particular gene expression

of the virus. The oncogene proteins in fact are kept at low levels throughout the initial life cycle, and the highly immunogenic products, the L1 and L2 capsid proteins, are synthesized only in the superficial layers of the epithelium [17,11].

Although HPV-related tumors are infiltrated by large amounts of macrophages that aggregate at the infection site after the recognition of transformed cells [18], their presence inside the microenvironment of solid tumors can have a part in disease progression. In fact, tumor-associated macrophages promote cancer cell proliferation and migration, angiogenesis and restriction of immune defenses [19], thus resulting in the disruption of the basement membrane, tumor growth, and metastasis.

During the immune response against pathogens, the balance between Th1 and Th2 must be sustained invariably in order to face intracellular or extracellular attacks, but this delicate interplay between the two phenotypes is distorted in HPV lesions. The HPV, as an intracellular enemy, should evoke Th1 immune response, but it appears that, in patients with intraepithelial and invasive cervical HPV lesions, the Th2 cytokine profile is prevalent. It is important to note that the reduced Th1 and increased Th2 responses lead to the suppression of cellular immunity and lesion progression [20,21].

Finally, HPV has developed defenses against cytotoxic T cells. In particular, the E7 oncogene down-regulates the expression of an antigen peptide transporter, which has a pivotal role in associating the MHC class I molecule with the viral antigen, which results in the suppression of HPV antigens' presentation and evasion from the human cellular defense [22,23]. Also the E5 protein has been found to down-regulate MHC/HLA class I [24].

HPV16 and the neoplastic transformation

HPV16 has a specific tropism for the mucosal epithelium of the anogenital and oropharyngeal tracts, in which it induces lesions. These lesions can spontaneously regress, unless the infection is persistent, and can evolve in more severe stages, leading to the development of carcinomas (Fig. 5).

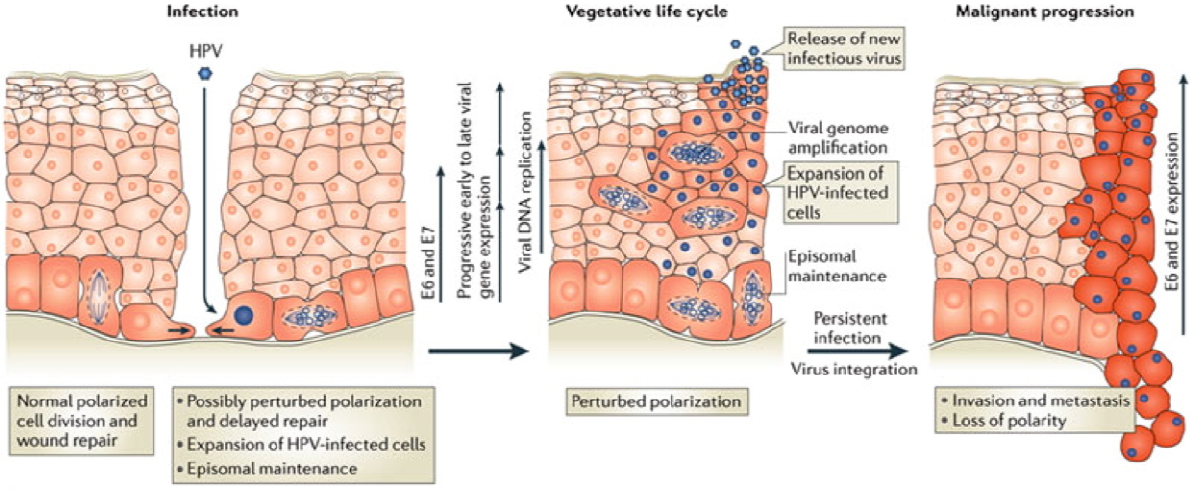

Fig. 5. HPV infection and malignant development. HPV infects the basal epithelial cells through microtraumas in the skin. In the vegetative life cycle, the coordinated expression of the viral gene products (from early to late) results in genome amplification, and, ultimately, to the generation of new infectious virus particles. Disruption of cell polarity in the basal cells and the promotion of aberrant cell division can contribute to a further expansion of infected cells that are capable of supporting viral genome amplification. Persistent infection with HPV over a number of years is linked to a much higher risk of the development of cervical cancer. This process is accompanied by frequent viral DNA integration into the host genome and an up-regulation in the expression of the E6 and E7 oncoproteins, resulting in the complete loss of polarity control and, ultimately, in invasion and metastasis.

The virus induces neoplastic transformation by the over-expression of the early E6 and E7 proteins, which are essential for the induction of the uncontrolled proliferation of the infected epithelial cells and lead to the malignancy [25]. The two oncogenic proteins cooperate in the induction of the neoplastic transformation by interacting with specific host cell targets involved in the regulation of the cell cycle and apoptosis [25,26].

In particular, E7, a small protein that is mainly localized in the nucleus [27], binds the oncosuppressive cellular Retinoblastoma protein (pRb) [28]. PRb can be differently phosphorylated during the cell cycle: in the G0 and G1 phases it is active and hypo-phosphorylated (pRb), whereas during the S, G2 and M phases it is hyper-phosphorylated (ppRb). In its active form, pRb is able to bind and inhibit the E2F/DP1transcriptional complex. This inactivates the binding of the complex to its promoter sequence and the following cell cycle progression and apoptosis are down-regulated [29]. The hyper-phosphorylated form is inactive and unable to bind the E2F transcriptional factor, and thus induces the transcription of the genes involved in the DNA synthesis and in the cell cycle progression [30,31]. In HPV16 infected cells, E7 binds and sequestrates pRb, allowing the constitutive expression of the genes regulated by E2F/DP1 transcriptional complex [31]. The HPV16 E6 oncogenic protein inhibits signals that induce the “programmed cell death”, which usually occurs after DNA damage or oncogene activation, and thus E6 mediates an anti-apoptotic activity. The most important E6 cell target is the p53 onco-suppressive protein, which usually regulates the expression of proteins involved in the cell cycle, such as the p21 cyclin-dependent kinase inhibitor, which, when expressed at high levels, induces cell cycle arrest and apoptosis [34]. In particular, E6 generates a trimeric complex with p53 and the ubiquitin protein-ligase E6AP [35]. After forming this complex, E6AP leads to the ubiquitination of p53 and its subsequent degradation by the 26S proteasome [35], which results in its drastic decrease and the consequent inhibition of its transcriptional functions. This mechanism determines the elimination of the check-points between the G1 and S as well as between the G2 and M phases of the cell cycle, that are normally regulated by the p53 expression [36]. Moreover, E6 carries out an anti-apoptotic activity by inducing the degradation of p53-indipendent cellular factors, involved in the adhesion, the cell polarity and the negative regulation of cell proliferation, such as hDLG, hScrib, hMAGI-

1 and MUPP1 [37,38]. E6 also induces cell immortalization by blocking the telomeres reduction, that normally represents an important signal for cell senescence [39,40].

The E6 and E7 oncoproteins have therefore a combined action: E7 increases p53 levels, leading to the cell cycle arrest or the apoptosis of infected cells, whereas E6, by inducing p53 degradation, inhibits the pro-apoptotic signals, allowing the progression of the infected cells in the cell cycle, and thus inducing the neoplastic transformation and immortalization (Fig. 6).

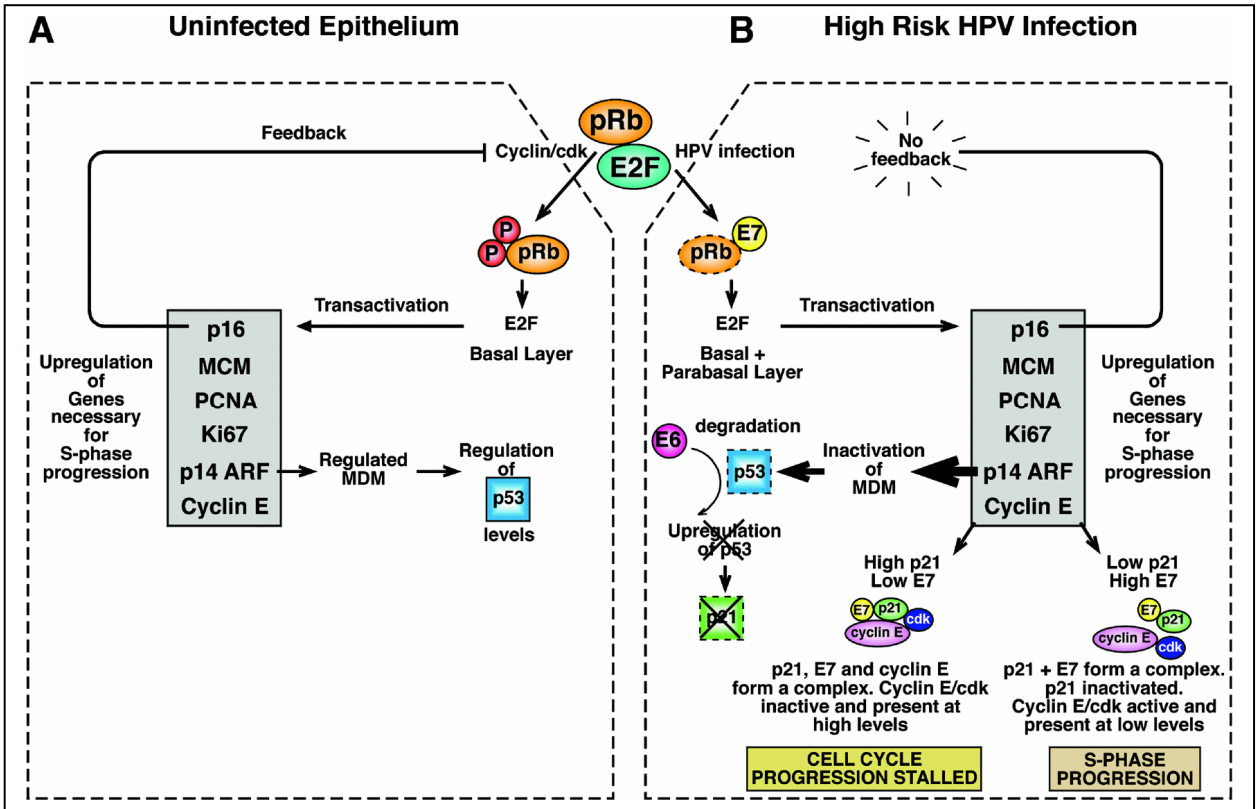

Fig. 6. Stimulation of cell-cycle progression by high-risk HPV types. HPV infection leads to deregulation of the cell cycle. Regulation of protein expression in uninfected epithelium is shown in (A). In the presence of high-risk HPV (B), the regulation of proteins necessary for cell proliferation is altered, allowing HPV to stimulate S-phase entry in the upper epithelial layers.
In cervical epithelium infected by high-risk HPV types, the progression through the cell cycle is not dependent on external growth factors, but it is stimulated by the E7 protein, which binds and degrades pRB and facilitates E2F-mediated expression of cellular proteins necessary for S-phase entry.
Cells respond to this stress by activating anti-oncogenic pathways, but these are counterbalanced by the E6 protein, which associates with the E6AP ubiquitin ligase in order to stimulate the degradation of the p53 protein and prevent growth arrest and/or apoptosis.

For tumor development, HPV16 must persistently infect the epithelial basal cells. When these cells replicate, the viral genome is distributed in both the daughter cells, which migrate into the granular layer where the differentiation process has begun [36]. In the uninfected tissue, the cells that leave the basal layer lose the nucleus and their capability to proceed in the cell cycle, but, thanks to E7 expression, the HPV-infected cells maintain their active state, proceed in the cell cycle [41] and rescue the nucleus during the migration along the epithelial tissue [36]. The transformed and immortalized basal cells generate daughter cells, which are unable to differentiate in keratinocytes, thus developing intraepithelial lesions of first grade (CIN1). These lesions, if not treated, can evolve in more severe forms (CIN2 and CIN3) with the invasion of the epithelium and the carcinoma development (Fig. 7).

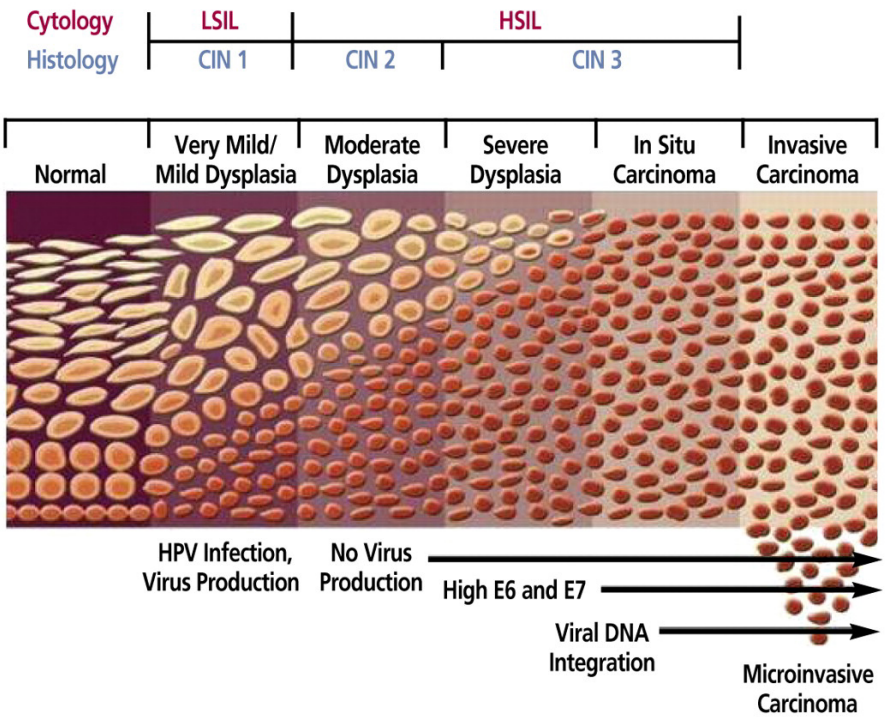

Fig. 7. Progression of persistent human papillovmavirus (HPV) infection in the cells of the cervix. A progression from normal to tumor cells is shown. The nucleus-to-cytoplasm ratio increases as cells progress through the stages of dysplasia and cancer.

HPV vaccines

To fight the HPV infection and the HPV-related cervical cancer, two immunization approaches can be pursued, a preventive strategy with the purpose of blocking the infection in still-healthy subjects, or a therapeutic strategy to clear the established infection and/or tumor already developed in infected subjects.

The preventive vaccines currently available are based on the capability of the viral L1 capsid protein of self-assemble in 72 pentameric capsomers, even in the absence of the L2 protein [42]. This event promotes the formation of empty capsids, the Virus-Like Particles (VLPs), which are structurally and immunologically similar to the virions, and, lacking the DNA genoma, completely safe [43].

At present, two preventive vaccines are commercialized and used globally: a quadrivalent HPV vaccine, produced by Merck & Co. Inc. (Gardasil®, Merck, Rahway, NJ, USA), and a bivalent HPV vaccine, produced by GlaxoSmithKline plc. (Cervarix™, GSK, Middlesex, UK). Gardasil® contains the VLPs from HPV type 6, 11, 16, and 18 and aluminum hydroxyphosphate sulfate as an adjuvant. Cervarix™ contains the VLPs from HPV type 16 and 18 and the AS04 adjuvant, which is a combination of aluminum hydroxide and monophosphoryl-lipid A (MPL) [44-47]. Unfortunately, preventive vaccines cannot control already established HPV infections, and the long delay before tumor development limits the assessment of their effects in lowering HPV tumor incidence over time. Although extensive screening for early diagnosis has led to a reduction in the mortality of women in developed countries, the 500,000 new cases of cervical cancer each year make the development of an effective therapeutic vaccine mandatory.

In a therapeutic setting, immunity can be utilized in two ways: by using specific natural or synthetic antibodies against defined targets, or by inducing an immune response in the host against specific antigens. In particular, for HPV-related lesions and cancer, viral antigens or/and virus-induced host antigens can be targeted by the following approaches.

Therapeutic antibodies

The use of intracellular antibodies (intrabodies) to inhibit protein functions holds promise for the treatment of human diseases. The difficulties associated with the development of intrabody-based therapies are similar to those that hamper the development of protein inhibitors in general, and reflect the problems encountered in the generation of reagents that are effective and specific.

For viruses that cause only local infections, such as HPV, the intrabody approach may be more appropriate. Intrabodies against E6 [48] and E7 [49] have been produced and proved to be effective in *in vitro* cancer cell models. More recently, an intrabody approach against E7 was proved to block tumor growth in animal models [50]. Given the accessibility of HPV-associated lesions to topical therapy, preclinical data suggest that large molecules, such as intrabodies, may be useful inhibitors of viral protein–protein interactions and particularly appropriate for the treatment of HPV-associated diseases.

Therapeutic vaccines

Therapeutic vaccines aim at eradicating or reducing HPV-infected cells by stimulating a specific cytotoxic T cell (CTL) response against infected target cells through the up-regulation of MHC Class I expression. Vaccine-mediated immune strategies could be directed towards at least two different stages: the viral infection and the established tumor.

Therapeutic vaccines should be able to eliminate the already-infected cells, and could be tailored on the presence of episomal or integrated viral sequences. In the first case, the vaccine targets could be all the early proteins, in the second case only E6 and E7 appear to be the realistic targets of intervention [51]. To this aim, the E6 and E7 of high-risk HPV genotypes could represent a good target for the immunotherapy, since they are expressed in 90-95% of cervical tumors and they are responsible for the cell transformation and progression of pre-neoplastic lesions to neoplasias [50,52].

Protein/peptide-based vaccines

To date, several protein or peptide-based vaccines are in development or undergoing clinical evaluation. A major limitation to peptide-based vaccines is the HLA restriction that can be overcome by the use of whole-protein-based vaccines harboring multiple immunogenic epitopes that can bind various allelic HLA molecules. On the other hand, protein-based vaccines predominantly elicits antibody rather than CTL responses, as proteins are processed intracellularly through the endocytic/MHC class II pathway. In addition, peptides and proteins administered without an adjuvant are poorly immunogenic. Therefore, most researches were focused on immunogens co-expressing chemokines, cytokines, and co-stimulatory molecules or co-administered with saponin-based [53] or liposome–based (LPD) formulations [54] and TLR agonists [55] as adjuvants to enhance vaccine potency. Peptide-based vaccines need to increase not only their low level of immunogenicity but also the obstacle of MHC restriction. To this aim TLR agonists have been explored as adjuvants for peptide-based HPV vaccines because of their capability to activate both innate and adaptive immunity and vaccines consisting in CTL and or TH epitopes, adjuvanted with TLR9 [56], TLR4 [57] and TLR3 [58] agonists, demonstrated their efficacy in mouse models.

DNA based genetic vaccines

Although DNA expression plasmid vaccines do not appear to induce as vigorous immune responses as live viral vaccine vectors, they have several advantages. Mainly, naked DNA is relatively safe, stable, cost efficient, and able to sustain reasonable levels of antigen expression within cells. Many strategies have been employed to produce an efficient delivery of targeted antigen to antigen-presenting cells (APC) such as dendritic cells (DCs), an enhancement of antigen processing and presentation, and an increase of DC and T cell interaction [59]. Recently, it has been reported that the fusion of the E7 gene of HPV16 with a plant virus coat protein produced a strong antitumor activity in the mouse model by activating both CD4+ and CD8+ T cells [60,61]. Similar results were obtained by fusion of the E7 gene to a gene encoding the mutated form of the immunotoxin from saponaria officinalis, the saporine [62]. Two different phase I clinical trials, examining the potential treatment of patients with anal dysplasia or with high-grade CIN, showed promising immunological responses [63,64]. An improved version of the vaccine, that includes HPV16 E7 and gene segments of E6 and E7 of HPV16/HPV18, is one of the few therapeutic vaccines reaching the phase II/III clinical trials in subjects with high-grade CIN. In a prospectively defined population of women younger than 25 years, CIN resolution was significantly higher in the vaccinated groups compared to placebo [65].

Bacterial/viral vectors

Bacteria, such as Listeria monocytogenes (LM) [66,67], Lactococcus lactis [68], Lactobacillus casei [69], Salmonella and Bacillus Calmette-Guérin, and several viral vectors, including Vaccinia Virus (VV), adenovirus, adeno-associated virus, alpha-virus, and their derivative vectors, have been used to deliver genes or proteins of interest to elicit antigen-specific immunotherapy. Among the bacterial vectors, LM has emerged as a

promising vector, as it is able to induce both CD8+ and CD4+ T-cell immune responses in animal models, to elicit regression of established tumors, and to overcome central tolerance by expanding low avidity E7-specific CD8+ T cells [66]. Among viral vectors engineered for the expression of HPV antigens, like adenoviruses [70,71], alpha-viruses [72-74] and VV [75-77], which was historically one of the first viral vectors employed in clinical trials in the therapy against HPV-associated cancers [78]. More recently, avipox viruses have been developed as novel vectors for the development of recombinant vaccines. Avipox viruses as canarypox (CP) and fowlpox (FP) might therefore represent safer immunogens, as they have demonstrated their activity in inhibiting the growth of HPV16 E7-expressing tumors in C57Bl/6 mice following a DNA-prime/fowlpox boost immunization protocol [79].

To date, many VV vaccines have been employed in clinical trials to deliver genes and antigens of interest efficiently. Phase I/II clinical trials in patients with vulvar or vaginal and early- or late-stage cervical cancers were already conducted with recombinant VVs encoding HPV16/HPV18 E6 and E7 antigens (TA-HPV) [78,80-82].

DC-based vaccines

The immune response to the initial stages of infection causes inflammatory responses that trigger innate effector cells, such as NK and NKT cells. This inflammatory response drives the innate immunity. The non-lytic nature of HPV infection also delays PAMP- and DAMP-induced inflammatory responses through TLRs and the inflammasomes. In the absence of inflammation, a number of events takes place that induce negative regulatory signals that can change the state of APCs by altering the expression of co-stimulatory molecules, and inhibit the induction of effector cytotoxic T cells. Therefore, a therapy aimed at reactivating these APCs could be a valid tool for clinical intervention.

Combinational immunotherapy

Given the importance of local microenvironment in the persistence of HPV lesions, strategies aiming at altering local immunity have shown some positive results. Therefore therapeutic HPV vaccine strategies have shifted toward combined approaches with radiotherapy and chemotherapy. Low-dose radiation in combination with HPV vaccination was effective in the treatment of tumors in preclinical models [83]. In particular, a chemotherapeutic agent in combination with DNA-based vaccines was proven as an effective HPV therapy in preclinical models [84-90].

Non-oncogenic $E6_{F47R}$ and $E7_{GGG}$ mutated proteins

Several studies suggest that E6 and E7 oncoproteins of high-risk HPVs are favorable targets to limit and inhibit the proliferation of cervical cancer cells due to their sustained expression in cancer tissues and their involvement in malignant transformation. The E6 inhibition was obtained either by decreasing the mRNA levels by RNA interference [91] or by transducing anti-E6 antibodies [92] or recombinant antibody fragments [48]. E6 mutants, defective for their binding to the p53 cellular target protein, have induced high levels of the oncosuppressor protein when introduced into HPV-positive cancer cells and senescence in HeLa cells [93], with a subsequent cell cycle arrest, sometimes leading to apoptosis.

$E6_{F47R}$ is a genetically mutated form of the HPV16 E6 protein, defective in its ability to lead the degradation of p53, because of the presence of a single-site mutation that determines the substitution of the phenylalanine in position 47 (F47) with an arginine (R) [94]. This mutation was introduced in a previous mutant, called E6 6C/6S, in which six cysteine residues were substituted with six serine to overcome problems related to the oxidation

and formation of aggregates that were shown during the HPV16 E6 recombinant protein production [95]. This mutant precursor, which maintained the oncogenic activity of the wild-type E6 protein, was modified to substitute different surface residues with the aim at verifying their effect on p53 degradation. The F47R mutation was the only substitution which showed to be able to inactivate the p53 degradation both on rabbit reticulocyte extracts, and on HPV-positive or -negative cells. Moreover, it was demonstrated that HeLa cells , which are derived from cervical cancer and constitutively express the HPV18 E6 protein, when transfected with $E6_{F47R}$ showed an increase in the levels of their endogenous p53 [94].

However, the native E6 protein presents the F47 amino acid residue completely exposed on the surface, and thus this mutation should not induce any change in the protein folding, although the presence of a positive charge on the introduced arginine generates electrostatic effects that alter the catalytic activity of the complex. $E6_{F47R}$ maintains the affinity for the ubiquitin ligase E6AP, and its ability to form the trimeric complex $E6_{F47R}$/E6AP/p53, but it lacks the capacity to induce the p53 polyubiquitination and degradation. The mutant protein is also able to have a negative dominant effect on the native E6 protein and is able to degrade the p53 *in vitro* as well as *in vivo*. In fact, in CaSki and SiHa cells, both HPV16-positive cell lines, the mutant protein can reactivate the p53 expression by a competitive mechanism against the endogenous native E6. When $E6_{F47R}$ is overexpressed, the $E6_{F47R}$/E6AP/p53 complex forms prevalently, instead of the complex with the E6 wild-type protein, leading to a reduction of the p53 degradation. The complex with the mutant protein also shows an increased half-life compared with the wild-type complex. Thus, the $E6_{F47R}$ prevents the cell growth and induces senescence in HPV-positive cells counteracting the oncogenic activity of the original E6 protein and behaving as an oncosuppressor [93].

The $E7_{GGG}$ protein is a mutant form of the HPV16 E7 oncoprotein, which was obtained by the introduction in the E7 gene of three single-site mutations in a region that represents the binding site to the target pRb oncosuppressor protein. These mutations induce the substitution of aspartate, lysine and glutamate amino acids with three glycine residues (GGG) in position 21, 24 and 26, respectively [96]. Each of these mutations can decrease or completely abolish the binding to the cellular pRb target protein, and the oncogenicity of native-E7. Moreover, it has been demonstrated that the E7 mutation inside the binding site significantly increases the immunogenicity of the protein [96]. In fact, the native protein suppresses the capacity to present the antigen by inhibiting DC differentiation [97], whereas $E7_{GGG}$ lacks this property and the antigen presentation is enhanced.

Poxviruses

Structure and replication

Poxviridae is a family of DNA viruses able to infect both vertebrates (subfamily *Chordopoxvirinae*) and invertebrates (subfamily *Entomopoxviridae*). Poxviruses are big sized (360 x 270 x 250 nm), ovoid and brick-shaped (Fig. 8) with some differences depending on the genus, and a more complex morphology than the other viruses.

The external structure consists of an envelope with a double-lipid layer membrane where 8 polypeptides are inserted. In the mature extracellular form, an additional outer membrane is usually present, which is absent in the intracellular forms and in the extracellular particles released by cell lysis. The envelope surrounds a dumbbell-shaped core, also called nucleoid, that consists of nucleoproteins with two associated structures, called lateral bodies, containing enzymes.

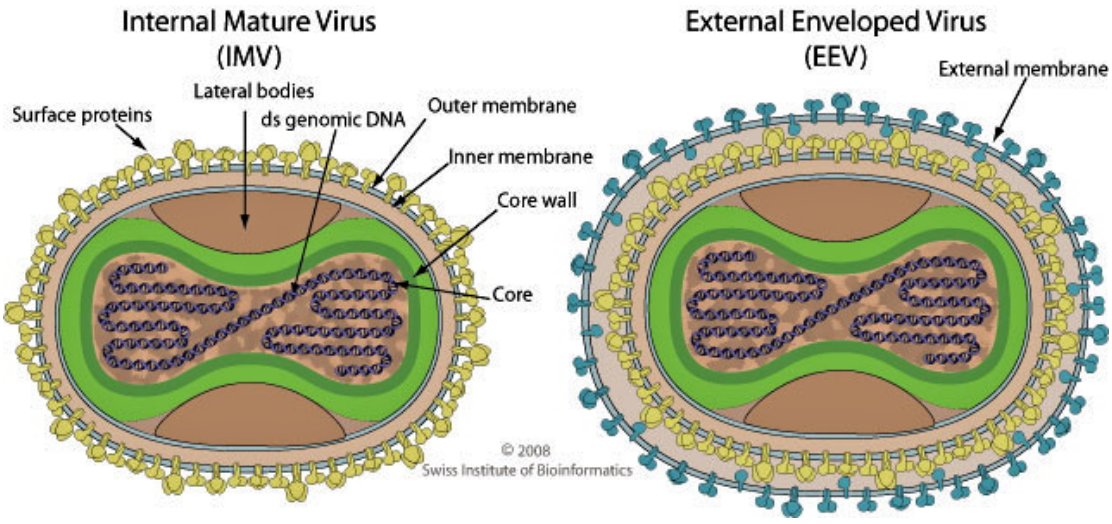

Fig. 8. Poxvirus virions. Schematic representation of the two distinct infectious viral particles: Intracellular Mature Virion (IMV or MV) and Extracellular Enveloped Virion (EEV or EV)

The poxvirus genome consists of a linear double-stranded DNA, with close ends, and a dimension comprised between 8.5 x 10^7 and 1.85 x 10^8 Da from parapoxviruses to fowlpoxviruses, respectively. The genome has a central region containing highly conserved genes involved in the viral replication, which can be distinguished in early, intermediate and late genes, on the basis of their activation during the viral replication. The two lateral regions include variable genes involved in the interaction with the host, and two Inverted Terminal Repetitions (ITRs) which consist of subsequent and repetitive, inverted sequences. Finally, two loops, called hairpin loops, link the two DNA lateral chains and are involved in the synthesis and desegregation of the DNA concatemer, that are transiently formed during the replication [98]. The viral particles contain enzymes for the DNA transcription and maturation (capping, polyadenylation and methylation).
Two different viral forms are able to infect the cell, the Intracellular Mature Virion (MV) and the Extracellular Enveloped Virion (EV), which has an additional outer membrane that is absent both in the MV and the extracellular virions released by cell lysis [99].

The poxvirus replication consists of several stages (Fig. 9) and begins with the binding to a cellular receptor on the host cell surface and the subsequent penetration and uncoating. The MV penetration occurs by fusion with the cytoplasmic membrane, whereas EV enter by endocytosis and fusion with the endosome membrane [100]. The following events include early genes transcription, DNA replication, late genes transcription, structural protein synthesis, maturation, assembly and release of the new viral progeny. During these stages, proteins coded in the previous step are required for the regulation of the genes that will be expressed during the following stage. Immediately after the infection, the viral mRNA transcription and maturation by capping, methylation and polyadenylation occurs inside the host cell. The early genes code for proteins that are expressed before

the DNA replication, whereas the late genes, coding for core and structural proteins and enzymes that will be included in the mature virions, are expressed after the replication of DNA. Finally, mature virions are released from the cell by exocytosis and cell lysis.

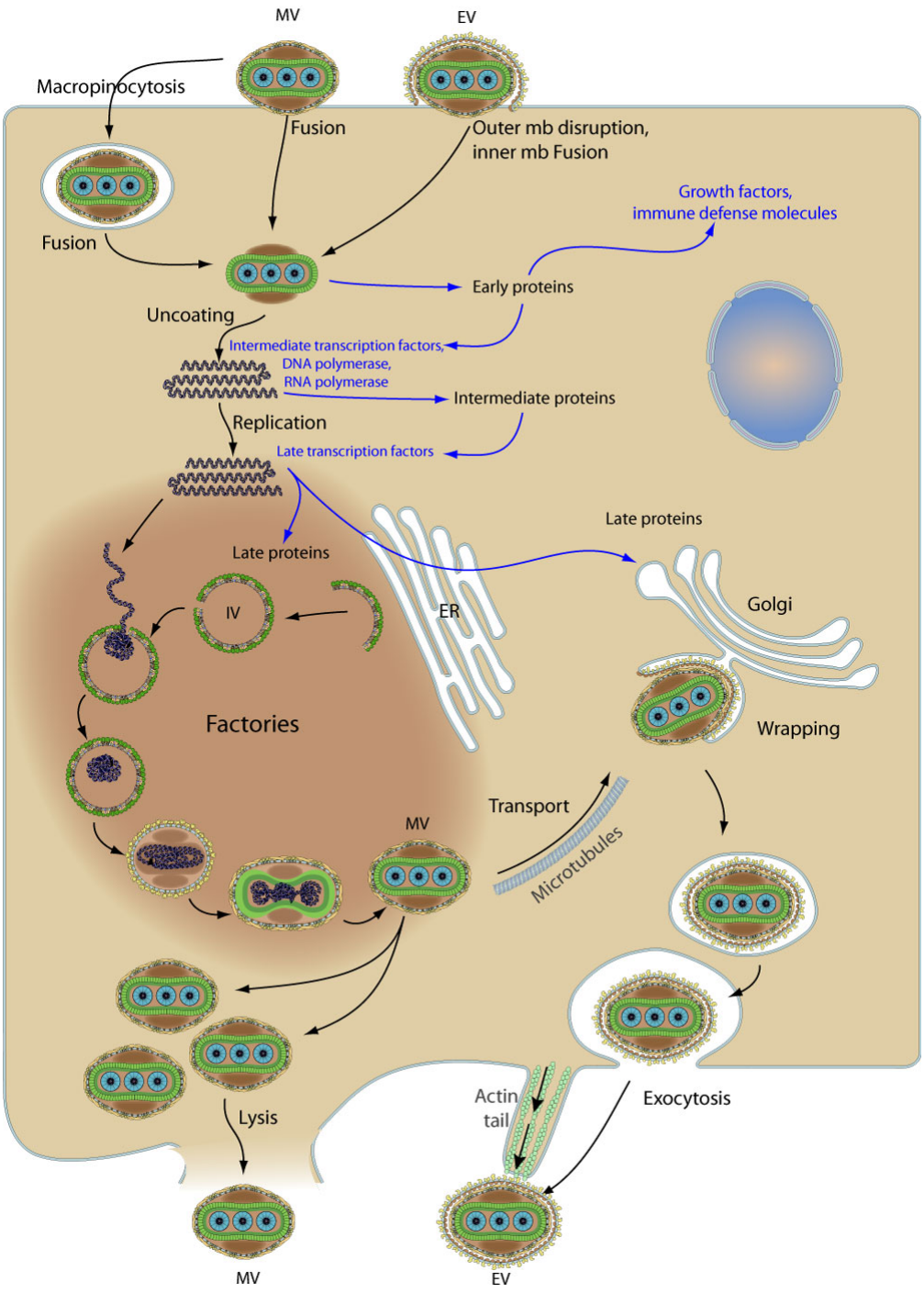

Fig. 9. Poxvirus replication. During the viral replication, both MV and EV can enter the cell by binding and fusion with the cell plasma membrane. After the viral uncoating, early genes are expressed that support viral DNA replication and modulate cellular functions. Products of intermediate genes tend to modulate intracellular functions. At the later stage, late gene products are produced to induce viral particles assembling. After assembling in the factories, virions are released from the host cell by lysis or exocytosis.

Fowlpox virus as a vaccine vector

The FP virus belongs to the family of *Poxviridae* and to the genus of *Avipoxvirus*, and, in its attenuated form, is used to prevent the corresponding poxvirus pathology in the poultry. The FP genome is a double-stranded DNA of 288 kbp, containing around 260 genes, and consists of a central region sided by two Inverted Terminal Repeats that have transcriptional promoter functions [101] (Fig.10).

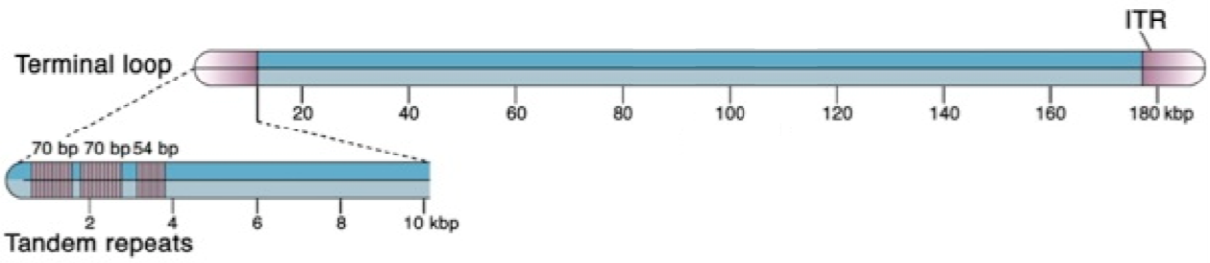

Fig. 10. Fowlpox virus genome. The genome of the fowlpox virus consists of a double-stranded and linear DNA, flanked by inverted terminal repeat (ITR) sequences, which are covalently-closed at their extremities.

Different promoter sequences are involved in the regulation of proteins expression during the early and late stages of the viral replication.

Like the other poxviruses, the FP virus has a unique transcriptional element that is recognized by the viral RNA polymerase. After specific deletions, its genome dimension allows the insertion of several exogenous genes, and, if the insertion occurs inside non-essential genes, its replicative ability is maintained. As a poxvirus, FP exclusively replicates in the cytoplasm, thus limiting the recombination events between the viral and host cell genomes, and the safety concerns shown by the other viral vectors used for

vaccine development. In particular, the FP virus, as well as the CP virus, although able to infect non-avian hosts, show an abortive replication in mammalian cells [102]. For these reasons, when used as a vector, the FP virus behaves as a live-attenuated vaccine capable to express the foreign genes and allows a proper processing of the antigens, that are presented in their native conformation in association with both class I and class II MHC molecules [103,104].

Some research groups have developed FP recombinants, that have demonstrated to express heterologous viral antigens, such as the 67 kDa glycoprotein of the Rabies Virus [105,106] and different proteins of the Human Immunodeficiency Virus [103,104,107-109]. Since the immunization with FP recombinants elicit very low levels of immune responses to the vector, it is possible to perform multiple boosts, and thus enhance the protective efficiency [110,111]. Moreover, the FP virus does not immunologically cross-react with the vaccinia virus, and can thus be administered also in subjects previously vaccinated against smallpox [112]. The abortive replication in mammal cells, its low pathogenicity and its ability to synthesize the antigens for a long time (up to 3-4 weeks) [113] allow a safe and continuous stimulation of the immune system [114], able to induce both an innate immune response, by activating natural killers cells and macrophages, and a specific immune response, by inducing B and T lymphocytes [115]. Finally, the FP genome size allows the insertion of multiple genes with a dimension up to 25 kbp [116-118], thus permitting the use of a unique recombinant vector against different pathogens. Not last, the genetic stability, the low-cost of production and the safety of this virus represent important features that enable FP to be used as an optimal vector for the production of Virus Like Particles (VLPs), expressing proteins in their native conformation.

AIM OF THE STUDY

The cervical cancer is one of the most important causes of morbidity and mortality in women worldwide, with a high prevalence in developing countries, where the screening for the precancerous lesions are not so much diffused.

The development of vaccines represents an improvement for the prevention of the disease, but the high numbers of newly infected subjects occurring each year, the impossibility to vaccinate already infected subjects as well as the difficulty to assess the duration of the vaccine protection, still represent a problem.

During the last years, research efforts have been focused on the development of therapeutic vaccines to treat already established HPV-induced precancerous lesions and carcinomas.

The aim of this project has been the construction and evaluation of two novel FP-based viral recombinants able to express a mutated, non-oncogenic, form of the E6 and E7 proteins of HPV16.

To this purpose, the $E6_{F47R}$ and $E7_{GGG}$ genes were separately inserted into DNA expression plasmids as well as into recombination plasmids with the aim of generating viral FP recombinants by a site-specific *in vitro* recombination in permissive CEFs. The viral recombinants were then selected by plaque purification, amplified and analyzed to assess their ability to express correctly the heterologous genes in different cell lines.

Finally, genetic and viral recombinants were involved in mice immunization by prime/boost protocols to evaluate their capability to stimulate specific immune responses, and therapeutic efficacy against already established HPV-induced tumors in the mouse model.

MATERIALS AND METHODS

Cell lines

CEF

Specific-pathogen-free primary Chick Embryo Fibroblasts (CEFs) were used for the production and titration of the FP wild-type virus (FPwt) and the viral recombinants ($FPE6_{F47R}$ and $FPE7_{GGG}$) as well as for the evaluation by RT-PCR, Western blotting and immunofluorescence, the expression of the $E6_{F47R}$ and the $E7_{GGG}$ proteins. CEFs were grown in Dulbecco's Modified Essential Medium (DMEM, Gibco, Life Technologies, Rockville, MD) supplemented with 5% heat-inactivated calf serum (CS, Gibco), 5% Triptose Phosphate Broth (Difco Laboratories, Beckton Dickinson, NJ), and 100 U/mL penicillin and 100 mg/mL streptomycin (P/S, Sigma Aldrich, Milan, Italy).

CEFs were prepared using specific-pathogen-free 11 day olds chicken eggs. Briefly, after disinfection with iodate-alcohol and incision of the eggshell, the embryo was extracted and the head and internal differentiated organs were removed. The remaining tissues were washed with Phosphate Buffered Saline calcium and magnesium-free (PBS^-), and then mechanically fragmented. After sedimentation, the fragments were washed three times with PBS^-, and dissociated by enzymatic digestion by adding one volume of 0.25% trypsin (Sigma) in PBS^- and gently stirring at 37 °C for 15 min. The digestion was blocked by incubating at 4 °C for 5 min, and the supernatant was filtered through sterile gauze. The cell sediment was repeatedly digested enzymatically until the complete disaggregation of the tissues occurred. The cell suspension was then diluted with an equal volume of growth

medium, and centrifuged at 2,800 x g for 15 min. After removal of the supernatant, the cell pellet was resuspended in growth medium and cells were distributed in Petri dishes.

Vero and MRC-5

Green monkey kidney (Vero) cells and normal human lung fibroblasts (MRC-5) were used to detect the expression of $E6_{F47R}$ and $E7_{GGG}$ proteins by the $FPE6_{F47R}$ and $FPE7_{GGG}$ recombinants in RT-PCR, Western blotting and immunofluorescence assays. Vero cells were grown in DMEM supplemented with 10% CS and P/S, whereas MRC-5 cells were grown in DMEM supplemented with 10% heat-inactivated fetal calf serum (FCS, Gibco) and P/S.

CaSki

CaSki cells, which contain multiple copies of the integrated HPV16 genome, were used as a positive control for E6/E7 protein expression, whereas the CaSki lysates were used as plate-bound antigens for ELISA assays. CaSki cells were grown in DMEM supplemented with 10% CS and P/S.

TC-1*

TC-1 star (TC-1*) mouse cells, which constitutively express the HPV16 E6 and E7 oncoproteins, and are able to induce tumors in 100% of mice [60], were used to induce tumors in the vaccinated animals. TC-1* cells were kindly provided by T.C. Wu (J. Hopkins Med. Ins., Baltimore, MD) and were grown in DMEM supplemented with 10% CS and P/S.

All of the cells have been maintained at 37 °C with 95% relative humidity and 5% CO_2.

Plasmids

pQE30-E6-His and pQE30-E7-His

To produce the E6 and E7 proteins, which were used as controls, plasmids pQE30-E6-His and pQE30-E7-His, kindly provided by Dr. Giorgi (Istituto Superiore di Sanità, ISS, Roma, Italy) and containing the HPV16 E6 and E7 genes, were inserted into E. Coli JM109 competent bacteria.

The transformation of the competent bacteria was performed by heat shock. Briefly, to the bacterial suspension were added 50 µg of plasmid, incubated on ice for 30 min, transferred at 42 °C for 40 sec and again on ice for 2 min. Bacteria were then added of 450 µL of Luria-Bertani broth medium (LB, 1% Bacto Triptone, 0.5% Bacto Yeast Extract, 0.172 M NaCl) and incubated at 37 °C for 1 h. After determining the plasmid presence, one bacterial clone was grown and stored at -80 °C.

pFP

The pFP recombination plasmid (Fig. 11) contains a sequence derived from pUC19, and two segments of the 3- β-hydroxysteroid dehydrogenase-steroid isomerase (DH) gene, that allow the homologous recombination with the FPwt genome during the *in-vitro* recombination (IVR). The sequence of the VVH6 poxviral early-late promoter is also present between the two DH recombination arms, which determines the constitutive expression of the inserted gene, followed by a multicloning site (MCS) for the insertion of the exogenous gene of interest.

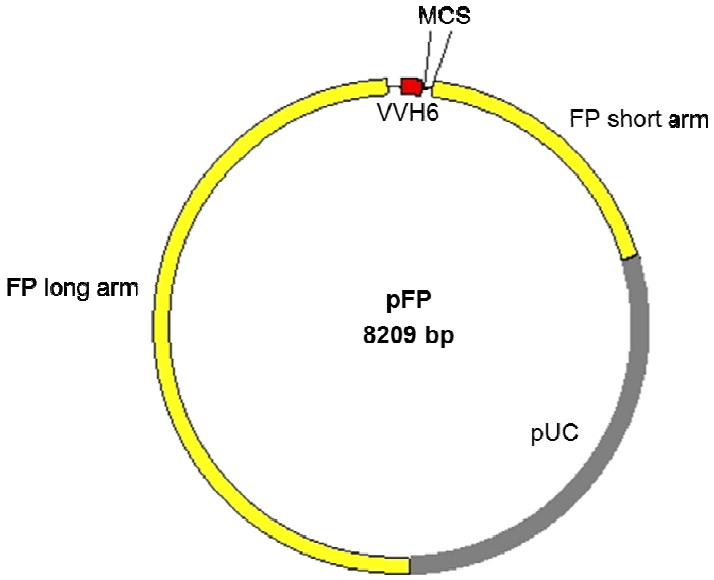

Fig. 11. Map of pFP recombination plasmid

pFPE6$_{F47R}$

The pFP$_{E6F47R}$ recombination plasmid (Fig. 12) was generated from pFP by insertion of the E6$_{F47R}$ gene down-stream of the VVH6 promoter. E6$_{F47R}$ gene was kindly provided by G. Travé (CNRS, University of Strasbourg, Illkirch, France). This mutant was obtained by replacing one phenylalanine (F) with one arginine residue (R), and six cysteine (C) with six serine (S) residues. The first mutation prevents the p53 degradation, and thus the oncogenic activity of the native E6 protein, whereas the C/S substitutions were introduced to minimize oxidation and stabilize the protein.

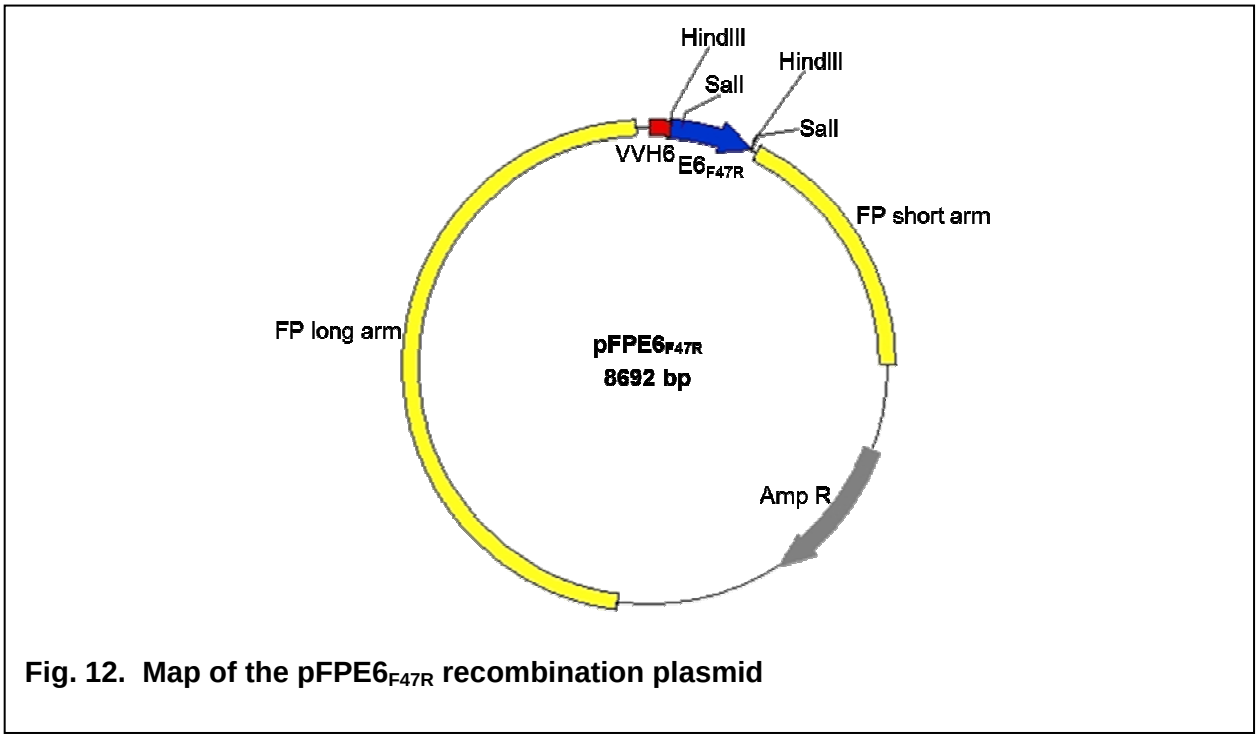

Fig. 12. Map of the pFPE6$_{F47R}$ recombination plasmid

pFPE6$_{F47R}$ construction

Amplification of the E6$_{F47R}$ insert

The E6$_{F47R}$ gene was amplified by PCR using pSG5-E6$_{F47R}$ as a template, the forward V364 (5’ ccg cgc ccg gga agc tta tgc acc aaa aga gaa ct 3’) and the reverse V99 (5’ cga agc ttt tac agc tgg gtt tct cta cg 3’) primers. The primers were designed to be complementary to the terminal sequences of the E6$_{F47R}$ gene, and to generate at the end sides two sequences containing restriction sites for the subsequent insertion of the heterologous gene into the vector. The PCR reaction was performed with 1 mM $MgCl_2$, 200 µM dNTPs, 1 µM primers and 0.025 U/µL Pwo polymerase (Boehringer Mannheim, Indianapolis, IN) in $MgSO_4$ buffer. The amplification was conducted using a Thermo Cycler PTC-200 (MJ Research, Walthman, MA) by initial denaturation at 94 °C for 1 min, followed by 30 cycles of 94 °C for 30 sec, 60 °C for 30 sec and 72 °C for 30 sec, and one final cycle at 72 °C for 7 min.

Subcloning of E6$_{F47R}$ in an intermediate vector

The E6$_{F47R}$ gene was first inserted in an intermediate pCR-BluntII-TOPO plasmid (Invitrogen, Carlsbad, CA) using the PCR-amplified sequence. The ligation was performed for 30 min, and was followed by bacterial transformation using the Zero Blunt TOPO PCR Cloning Kit (Invitrogen). This last plasmid, which has blunt ends bound to the Vaccinia Virus topoisomerase I, has allowed an easy insertion of the DNA fragment into the intermediate vector.

The bacterial suspension was seeded on Petri dishes containing LB agar in the presence of kanamycin (50 µg/mL) and incubated at 37 °C overnight (o/n). Some bacterial clones were transferred for 1 h in 1 mL of LB for a PCR pre-screening, with the aim at verifying

the presence of the insert, by using the forward V226 (5’ gta aaa cga cgg cca g 3’) and reverse V227 (5’ cag gaa aca gct atg ac 3’) primers, which are complementary with the plasmid vector. After initial denaturation at 94 °C for 4 min, the amplification was performed by 35 cycles at 94 °C for 30 sec, 50 °C for 30 sec and 72 °C for 45 sec, and one final cycle at 72 °C for 7 min. PCR-positive clones were added of 1 mL LB, incubated at 37 °C o/n, and the plasmid DNA was extracted by the minilysates method. Briefly, the bacterial suspension of each clone was centrifuged at 25,000 x g for 1 min, and, after removing the supernatant, the pellet was resuspended in 200 µL of cold buffer (50 mM Tris HCl, 10 mM EDTA, pH 8). The suspension was added of 200 µL of lysis buffer (200 mM NaOH and 1% SDS) and incubated at room temperature for 10 min. Then, 200 µL of CH_3COOK 3 M (pH 4.8) were added to precipitate the cell debris, and the mix was incubated at 4 °C for 5 min. After centrifugation at 16,000 x g and incubation at 4 °C for 10 min, the supernatant, containing the plasmid DNA, was added of 1 mL of 100% EtOH. A further incubation was then performed at room temperature for 10 min, followed by a centrifugation at 25,000 x g and 10 min at 4°C. The precipitated DNA was washed with 500 µL of 70% EtOH, dehydrated and resuspended in bidistilled water.

The presence of the insert was confirmed by digesting enzymatically the plasmid DNA with HindIII (Fermentas, M-Medical, Milan) and EcoRI (Fermentas) using 4 U/µg of DNA, and incubating at 37 °C for 1 h. The obtained fragments were separated by electrophoresis on 0.8% agarose gel. Finally, one positive plasmid clone was chosen and sequenced (Genenco, M-Medical, Milan) to verify the absence of any mutations, which could be introduced into the sequence after PCR amplification. The plasmid, called pCR-BluntII-TOPO/$E6_{F47R}$, was amplified, purified by the mini extraction kit (Qiagen, Hilden, Germany), and used for cloning.

Cloning of $E6_{F47R}$ into the pFP recombination plasmid

The pCR-BluntII-TOPO/$E6_{F47R}$ plasmid was cut with HindIII (Fermentas), and after separation on 0.8% agarose gel, the $E6_{F47R}$ gene (477 bp) was ligated with the pFP plasmid vector, previously linearized with the same restriction enzyme. The linearized pFP vector was dephosphorylated by using 1 µg of DNA/1 U of alkaline phosphatase (CIP, Boehringer Mannheim) at 37 °C for 30 min, to avoid self-ligation events. After inactivation of the enzyme at 65 °C for 15 min, the vector was resuspended in bidistilled water.
The insertion of $E6_{F47R}$ gene in the pFP plasmid was performed by using 100 ng of insert and an insert:vector molar ratio of 3:1, and 1 U of T4 DNA ligase (USB, Cleveland, OH) in a final volume of 10 µL. The ligation reaction was performed at 16 °C o/n, and the ligation product was used to transform DH5α competent bacteria by heat shock, as already described. Aliquots of bacteria were seeded in Petri dishes containing agarized LB and ampicillin (100 µg/mL), and incubated at 37 °C o/n. Some bacterial clones were transferred in liquid growing media and incubated at 37 °C for 1 h, and the presence of the insert was evaluated by PCR using the forward V62 (5' ggt tct tga ggg ttg tgt 3') and the reverse V104 (5' gga gat gcg atg ata aga gga 3') primers, which are complementary to the pFP plasmid sequences. The amplification was performed by denaturation at 94 °C for 4 min, followed by 30 cycles at 94 °C for 30 sec, 52 °C for 30 sec and 72 °C for 1 min, and one final cycle at 72 °C for 7 min. A few positive clones were added of 1 mL of LB with ampicillin and grown at 37 °C o/n. The plasmid DNA was extracted from the clones by minilysates and analyzed by evaluating the fragments pattern obtained after enzymatic digestion. The presence and orientation of the gene was confirmed by digestion with HindIII and SalI (Fermentas), and separation of the fragments in 0.8% agarose gel. The plasmid DNA of one positive clone was sequenced to exclude the presence of mutations, and the obtained recombination plasmid was called pFPE6_{F47R}.

pFPE7$_{GGG}$

The pFPE7$_{GGG}$ recombination plasmid (Fig. 13) was generated by insertion of the E7$_{GGG}$ gene down-stream of the VVH6 promoter. PE7$_{GGG}$, kindly provided by Dr. A. Venuti (Istituto Nazionale Tumori Regina Elena, IFO, Roma, Italy), was obtained by substituting the DLYCYE motif (amino acids 21-26) that is located in the CR2 homology domain by the GLYGYG sequence. This mutation prevents pRb binding and oncogenicity of the E7 protein.

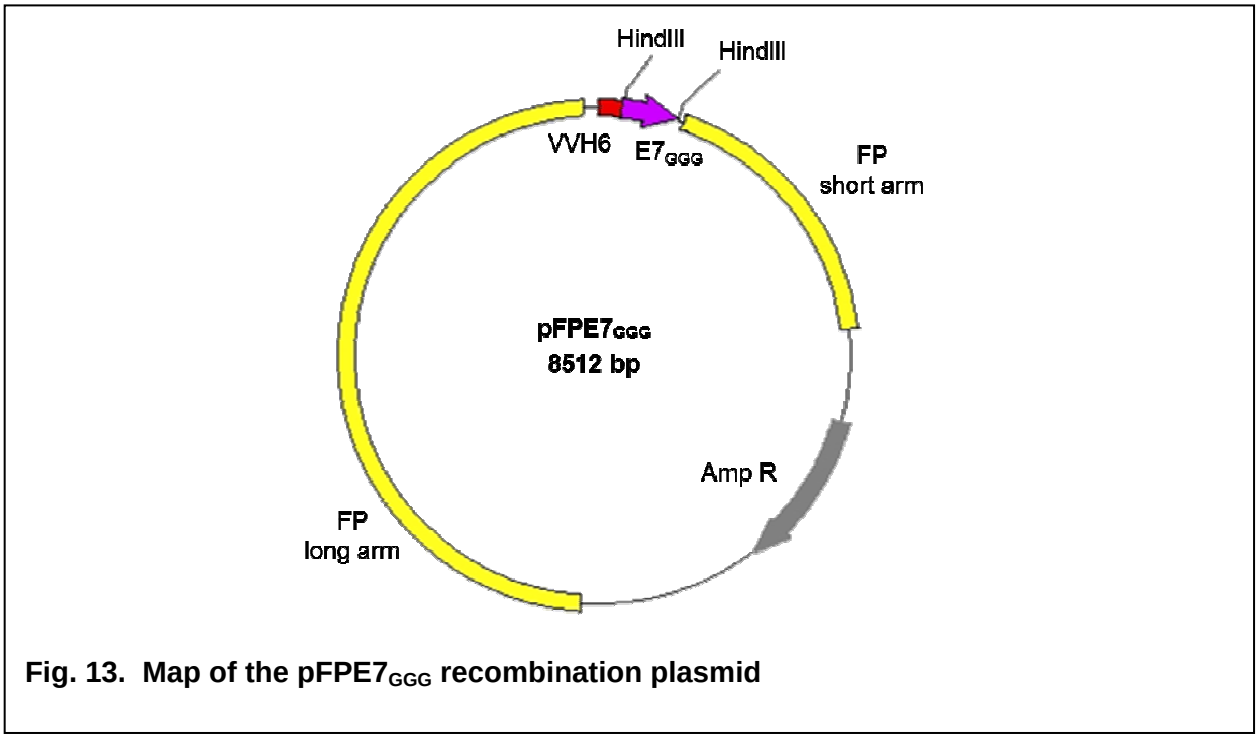

Fig. 13. Map of the pFPE7$_{GGG}$ recombination plasmid

pFPE7$_{GGG}$ construction

Amplification of the E7$_{GGG}$ insert

The E7$_{GGG}$ gene was prepared by PCR using the expression plasmid pcDNA3.1-E7$_{GGG}$ as a template. The amplification was performed using the forward V302 (5’ gcc gcg ccc ggg aag ctt atg cat gga gat aca cct aca tt 3’) and the reverse V303 (5’ gcc gcg gtc gac aag ctt tta tgg ttt ctg aga aca gat gg 3’) primers, which were designed to be complementary with the terminal regions of the E7$_{GGG}$ gene and to contain at the ends two enzymatic restriction sites and allow the subsequent insertion in the pFP vector. The PCR reaction was conducted at the final condition of 1 mM $MgCl_2$, 200 µM dNTPs, 1 µM primers and 0.025 U/µL Pwo polymerase in buffer containing $MgSO_4$, using the PTC-200 Thermo Cycler. The amplification was performed by denaturation at 94 °C for 1 min, followed by 24 cycles at 94 °C for 30 sec, 58 °C for 30 sec, 72 °C for 30 sec, and one final cycle at 72 °C for 7 min.

Cloning of E7$_{GGG}$ into the pFP recombination plasmid

The amplified E7$_{GGG}$ gene as well as the pFP plasmid vector were cut with HindIII (Fermentas) and incubated at 37 °C for 1 h. After buffer removal and resuspension in bidistilled water, the pFP vector was dephosphorylated, as already described, to avoid the occurrence of self-ligation events. The insertion of the E7$_{GGG}$ gene into pFP was performed by ligating 100 ng of E7$_{GGG}$ and an insert:vector molar ratio of 3:1 in the presence of 1 U of T4 DNA ligase (USB) in a final volume of 10 µL. The reaction was conducted o/n at 16 °C and the ligation product was used to transform DH5α competent bacteria, as already described. The seeding of bacteria, clones growth and isolation, and the subsequent plasmid DNA extraction was performed as already described. The presence and orientation of the insert was verified by PCR amplification using the forward

primer V62, which recognizes the promoter sequence, and the reverse V101 primer (5' cga agc ttt tat ggt ttc tga gaa cag 3'), which is complementary to a gene fragment. One positive clone was chosen sequenced to verify the absence of mutations and called pFPE7$_{GGG}$.

Plasmid DNA amplification and purification

The production of a high amount of plasmid DNA and its purification was performed by alkaline lysis followed by ultracentrifugation on a CsCl gradient. Briefly, bacterial clones containing the right plasmid, were grown at 37 °C o/n in 750 mL of LB medium in the presence of ampicillin (100 µg/mL), and centrifuged at 6,000 x g at 4 °C for 10 min. The pellet was resuspended in 18 mL of cold STE buffer (50 mM glucose, 25 mM Tris HCl pH 8 10 mM EDTA), added of 42 mL of lysis buffer (100 mM NaOH and 10% SDS), and incubated at room temperature for 5 min, and then, at 4 °C for 5 min. The mix was added of 21 mL of cold 3 M CH_3COOK, pH 4.8, incubated at 4 °C for 10 min, and bacteria debris were sedimented by centrifugation at 7,000 x g at 4 °C for 10 min. The supernatant, filtered on gauzes, was added of 0.6 volumes of isopropanol to precipitate the DNA, and then centrifuged at 7,000 x g at 4 °C for 15 min. To obtain a purified plasmid DNA, the pellet was resuspended in 3.8 mL of TE buffer (10 mM Tris HCl and 1 mM EDTA, pH 8), added of 1 g/mL of CsCl and 2.5 mg/mL of EtBr, and then ultracentrifuged on NVT-90 vertical rotor (Beckman Coulter, Milan, Italy) at 60,000 rpm at 24 °C o/n. The plasmid DNA band, detectable using an UV lamp was extracted and washed three times using a volume of isopropanol. The aqueous phase, containing the DNA, was added of 2 volumes of TE buffer and 1.5 volumes of 100% EtOH, to allow the plasmid DNA precipitation, and then centrifuged at 10,000 x g at 4 °C for 30 min. Finally, the pellet was washed with 70% EtOH, drayed and resuspended in bidistilled water.

pcDNA3E6$_{F47R}$ and pcDNA3.1E7$_{GGG}$

The pcDNA3E6$_{F47R}$ and pcDNA3.1E7$_{GGG}$ expression plasmids, containing the same mutated E6$_{F47R}$ or E7$_{GGG}$ sequences of pFPE6$_{F47R}$ or pFPE7$_{GGG}$ recombination plasmids, were used for the mice immunizations. These plasmids were generated from pcDNA3 or pcDNA3.1 (Life Technologies Corp., Carlsbad, CA, USA), by insertion of the E6$_{F47R}$ or E7$_{GGG}$ genes. They were propagated in E. coli and extracted by alkaline lysis, followed by purification and endotoxin removal (Qiagen, EndoFree Plasmid Giga Kit).

Virus

Construction of the $FPE6_{F47R}$ and $FPE7_{GGG}$ recombinants

The viral recombinants were generated by homologous site-specific recombination, which occurred between the FPwt and the $pFPE6_{F47R}$ or $pFPE7_{GGG}$ recombination plasmids. During this event, the DH gene is inactivated by the insertion of the heterologous gene downstream of the VVH6 promoter.

Site-specific homologous In Vitro Recombination (IVR)

The recombination events occur between the FPwt DNA genome and the DNA recombination plasmid in the cytoplasm of CEFs, after viral infection and plasmid transfection by the $CaPO_4$ method (Fig. 14).

To this aim, CEFs grown to sub-confluency were washed once with PBS^-, infected with the FPwt virus (5 pfu/cell) in a final volume of 200 µL of Earle's Balanced Salt Solution (Earle's BSS) and incubated at 37 °C for 1 h. After the incubation, cells were added of growing medium supplemented with 2.5% CS and 2.5% TPB, and incubated for 3 h.

The transfection was performed after preparing 62.5 µg plasmid, and 125 mM $CaCl_2$ in HEBS pH 7 (20 mM Hepes, 150 mM NaCl, 0.7 mM Na_2HPO_4, 5 mM KCl and 6 mM dextrose) in a final volume of 500 µL, which was maintained at room temperature for 30 min. After incubation, the transfection solution was distributed onto the infected CEFs, and left at 37 °C for 3 h. Cells were then washed once with PBS^-, and DMEM supplemented with 2% FCS was added. Cells were maintained in incubator for 48 h, and then lysed by freeze/thawing three times. Cell debris were removed by centrifugation at 2,000 x g at 4 °C

for 15 min, and the supernatant, diluted from 1:1,000 to 1:3,000 with growing medium, was used to infect confluent CEFs for 1 h at 37 °C. After infection, each Petri dish was added of 5 mL of DMEM supplemented with 2.5% CS, 2.5% TPB and 0.7% LE agarose (SeaKem, FMC BioProducts, Rockland, ME). At the third day post infection (p.i.), each Petri dish was added of additional 5 mL of agar medium supplemented with 1.5% Neutral Red (RN, Gibco), for a better visual detection of the viral plaques of lysis.

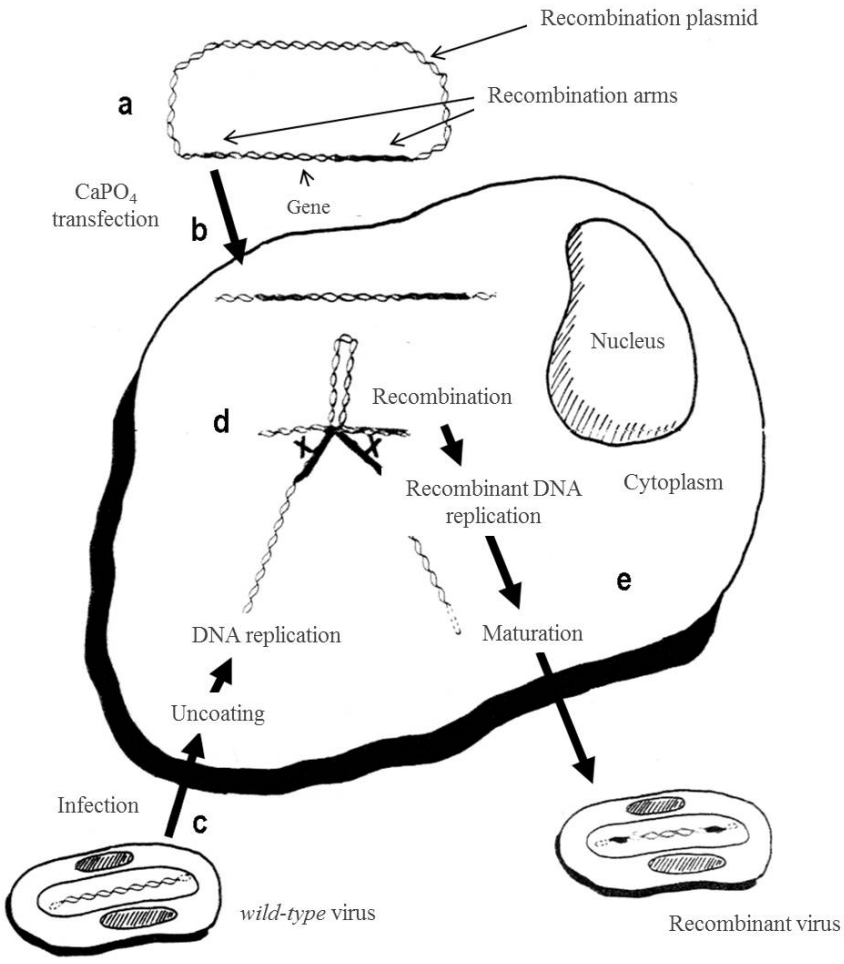

Fig. 14. *In-Vitro* Recombination (IVR)
Schematic representation of the events occurring during the IVR. Briefly, the presence of the recombination plasmid and the genome of the wild-type virus inside the same cell leads to the site-specific transfer of the heterologous gene into the genome of the wild-type virus and allows the formation of a new recombination virus.

Purification of FP recombinants

To the aim of obtaining 100% pure clones of the viral recombinants, several rounds of plaque-purification were performed by the following passages:

- *Replica plating*

  The agarized medium was removed from the Petri dishes, and a nitrocellulose membrane (Protran BA 85 0,45 µm, Schleicher & Schuell, Whatman, NJ) was laid down on the cell layer. A 3MM paper filter (Whatman), pre-soaked of transfer buffer (50 mM Tris HCl, pH 7.5 and 150 mM NaCl), was gently positioned over the membrane to allow the transfer of the virus onto the nitrocellulose membrane. Filter and membrane were removed from the dish and a second nitrocellulose membrane, defined “replica membrane”, was laid down on the first membrane, defined “original membrane”. The original membrane was used to detect the recombinant viral plaques by hybridization with a ^{32}P-labelled probe, whereas the replica membrane was stored at -20 °C and used to isolate the positive plaques, which were detected as a spot onto a radiography film.

- *Membrane denaturation*

  The original membranes were denatured with 0.5 M NaOH, neutralized twice with Tris HCl 1 M pH 7.6, and twice with a solution of 100 mM Tris HCl, pH 7.6, and 150 mM NaCl. Each treatment was performed for 5 min, allowing the membranes to air-dry between a step and the other.

- *Preparation and* ^{32}P *probe labeling*

  The probes were obtained by PCR amplification of a gene fragment. In particular, for the $E6_{F47R}$ gene the forward primer V348 (5’ ctg caa tgt ttc agg acc 3’) and the reverse

primer V350 (5’ ata cat cga ccg gtc cac 3’) were used, whereas for $E7_{GGG}$ the forward primer V302 and the reverse primer V303 were utilized. The probes’ labeling was performed using the Multiprime labelling system (Amersham International, Buckinghamshire, UK), following the manufacturer’s instructions, and the ^{32}P-dCTP as a labelled nucleotide (Perkin Elmer, Norwalk, CT).

- *Hybridization and autoradiography*

  The denatured original membranes were incubated at 60 °C for 1 h in hybridization buffer (4X SSC, 0.1% SDS, 0.1% NaPP, 100 µg/mL salmon sperm DNA and 5X Denhardt solution), then added of the radiolabelled probe, and incubated o/n at the same temperature. The exceeding probe was removed by washing at 60 °C for 2 h using a washing buffer (0.2X SSC, 0.2% SDS, and 0.2% NaPP). Membranes were left on 3MM paper to air-dry and overlaid with a photographic film (Biomax MS, Amersham International) o/n at -80 °C before film development.

- *Isolation of the recombinant clones*

  The autoradiography-positive plaques were localized on the original membranes, detected on the corresponding replica membranes and excised using a cutter. Each plaque was transferred in 1 mL of Earle’s BSS containing 0.06% trypsin, incubated at 37 °C for 5 min and sonicated for 1 min using a Sonicator W-385 (Heat Systems-Ultrasonic, Inc, Farmingdale, NY) to allow the rescue of the recombinant clone. The viral suspension was then serially diluted 1:10 to infect confluent CEFs.

Amplification of recombinant clones

Some clones of FP recombinants, obtained by plaque purification, were amplified to the aim at verifying their ability to express the heterologous gene. CEFs were infected with 200 µL of virus suspension and after 4 days, cells were lysed by freeze/thawing three times, to allow the virus release. Cell debris were removed by centrifugation at 2,000 x g at 4 °C for 15 min and the virus supernatant was used to analyze the protein expression of the foreign gene before proceeding to the production of a high amount of the chosen viral clone.

Large-scale amplification of viral clones

The large-scale production was performed on confluent primary CEFs (1.4 x 10^7 cell/Petri dish). The cells were infected by using each viral clone (0.2 PFU/cell) diluted in a final volume of 500 µL Earle's BSS/Petri dish, and incubating at 37 °C for 1 h. At the end of the incubation, each Petri dish was added of 5 mL of DMEM supplemented with 2.5% CS and 2.5% TPB. When the cytopathic effect was detectable (5 days p.i.), cells were harvested using a rubber cell harvester, centrifuged at 30,000 x g at 4 °C for 1 h, and pellets were frozen at -80 °C. After thawing, pellets were treated with 0.06% trypsin in TNE (0.001 M Tris, 0.15 M NaCl, 0.001 M EDTA, pH 7.4) at 37 °C for 5 min. To allow the viral release from the pellet, cells were sonicated several times (20 sec for 3-5 times). For purification, the viral suspension was layered onto a discontinuous sucrose gradient (30-45% w/w) and ultracentrifuged at 38,000 x g at 4 °C for 1 h. The viral band at the interface was harvested, washed with 0.001 M Tris HCl pH 9, and sedimented at 67,000 x g at 4 °C for 1 h to remove the residual sucrose,. The viruses were resuspended in PBS^-, sonicated, aliquoted, stored at -80 °C, and titred.

Virus titration

The evaluation of the recombinant concentration was performed by viral titration, in duplicate on confluent CEFs grown into 5-cm-diameter Petri dishes. Cells were infected with serial dilutions of the virus, from 10^{-3} to 10^{-8}, in 200 µL of Earle's BSS, incubated at 37 °C for 1 h, and then, added of agar medium (5 mL/Petri). After 3 days, a new layer of agar medium was added (5 mL/Petri), supplemented with NR. After 24 h, the plaques were counted where the virus dilution allowed the optimal plaque separation. The viral concentration was expressed as PFU/mL and worked out by the formula: mean of the plaque numbers x dilution factor x volume conversion factor.

Assays

mRNA isolation

The mRNA was extracted from infected-cells (CEF, Vero and MRC-5) by Trizol LS (Gibco). Cells were infected (1 PFU/cell) with the different viral recombinants and, after a 24-h incubation, the mRNA extraction was performed following the manufacturer's instructions. Briefly, cell samples, stored at -80 °C, were thawed and added of 80 µL of chloroform. After incubation at 4 °C for 5 min and centrifugation at 12,000 x g at 4 °C for 15 min, the aqueous phase, containing the RNA, was transferred in new RNase-free tubes. The RNA was precipitated by adding 2 volumes of isopropanol, incubated at 4 °C for 15 min, and then centrifuged at 12,000 x g at 4 °C for 15 min. The RNA pellet was washed with 70% EtOH, centrifuged at 7,500 x g at 4 °C for 5 min, drayed and resuspended in water treated with dietilpirocarbonate (DEPC). Samples were stored at -20 °C o/n, and then analyzed by electrophoresis separation in 1% agarose gel to evaluate the integrity of the bands, and by spectrophotometer to assess the RNA concentration.

RT-PCR

The $E6_{F47R}$ and $E7_{GGG}$ gene expression was evaluated by RT-PCR of RNA samples extracted from infected cell lines. The amplification was performed by the Access RT-PCR System (Promega, Madison, WI) using 50 ng of RNA in a final volume of 10 µL. The amplification of the $E6_{F47R}$ transcript was performed by using the forward primer V348 (5' ctg caa tgt ttc agg acc3') and the reverse primer V99 (5' cga agc ttt tac agc tgg gtt tct cta

cg 3'). For the $E7_{GGG}$ transcript, the forward V302 and the reverse V303 primers were used. The RT-PCR reaction was performed at the final condition of 200 µM dNTPs, 1 µM primers, 0.1 U/µL AMV reverse-transcriptase, 0.1 U/µL TLF DNA polymerase. $MgSO_4$ was used at 2.5 mM for $E6_{F47R}$, and at 2 mM for $E7_{GGG}$. The reverse-transcription step was conducted at 48 °C for 45 min, followed by a step at 94 °C for 2 min to inactivate the enzyme. The $E6_{F47R}$ amplification was performed by 40 cycles at 94 °C for 30 sec, 55 °C for 30 sec and 68 °C for 30 sec, whereas for $E7_{GGG}$ the annealing and the extension were performed at 58 °C for 30 sec and 68 °C for 30 sec, respectively. The final cycle was performed at 68 °C for 7 min.

RNAs extracted from mock- or FPwt-infected cells were used as negative controls, whereas RNAs extracted from pSG5-$E6_{F47R}$ or pcDNA3.1-$E7_{GGG}$ plasmid-transfected cells were used as positive controls.

SDS-PAGE and Western blotting

CEF, Vero and MRC-5 confluent cells were infected with the different viral recombinants or with FPwt (10 PFU/cell) and incubated at 37 °C for 1 h. After infection, 5 mL/Petri dish of DMEM supplemented with 2.5% CS were added to each dish and cells were incubated at 37 °C o/n. Cells were washed once with PBS^-, lysed with 300 µL/Petri of 2X lysis buffer (0.05 M Tris HCl, 2% SDS, 10% glycerol, 1.5% DTT, 0.05% bromophenol blue) and harvested. After an incubation at 100 °C for 5 min, cells were cooled at 4 °C to complete the lysis. Samples were loaded in a 15% acril/bisacrilamide gel and run at 40 mA for 3 h in cold buffer (0.025 M Tris HCl, 0.192 M glycine, 0.1% SDS). The separated proteins were transferred on a 0.2 µm nitrocellulose membrane (NC, Trans-Blot Transfer Medium, Bio-Rad Laboratories, Hercules, CA) for 1 h at 100 V in cold buffer and by using a transfer device (Trans-Blot Electrophoretic Transfer Mini Cell, Bio-Rad). The NC was then processed by treating with 0.5% glutaraldehyde (Polysciences, Warrington, PA) for 30 min with gentle stirring, followed by washing with distilled water and saturation for 1 h with a buffer containing 5% skimmed milk in PBS^- 1X. The NC was then washed shortly with wash buffer (0.1% Tween-20 in PBS^- 1X) and incubated at 4 °C o/n with the primary antibody diluted in 1% milk plus 0.1% Tween-20 in PBS^- 1X. The identification of $E6_{F47R}$ was performed using a 1:100-dilution of the mouse polyclonal AbE6/Gi primary antibody (C. Giorgi, Istituto Superiore di Sanità, Rome, Italy). For $E7_{GGG}$, a 1:100-dilutions of the ED17 (AbE7/SC, Santa Cruz Biotechnology, CA, USA) or the 8C9 (Zymed Lab., Invitrogen) monoclonal antibodies were used. After 30 min washing, the NC membranes were incubated at room temperature for 1 h with a 1:2,000-dilution of the goat anti-mouse horseradish peroxidase (HRP) conjugated secondary antibody (Dako Cytomation, Carpinteria, CA, USA) and washed for 2 h, before the proteins were revealed by the ECL

system (GE Healthcare, Buckinghamshire, UK). Cells infected with FPwt were used as a negative control, whereas the E6/E7 proteins, CaSki cell lysates or FPE7-infected cell lysates [119] were used as positive controls.

Immunofluorescence

CEF, Vero and MRC-5 cells were seeded in 6-well plates on cover glasses, previously dipped in 100% EtOH and flame-sterilized. After 16/18-h incubation, cells were infected (5 PFU/cell) with the different recombinants. FPwt-infected cells were used as a negative control, and, to detect the $E7_{GGG}$ expression, FPE7-infected Vero cells [85] were used as a positive control. After infection, cells were added of DMEM supplemented with 2% FCS and incubated o/n. After washing twice with PBS^-, cells were fixed with 2% formaldehyde (methanol-free ultrapure Formaldehyde, Polysciences) in PBS^-, incubated at room temperature for 10 min, at 4 °C for 5 min in wash buffer (0.2% BSA plus 0.1% NaN_3 in PBS^+ 1X), and then fixed again in cold acetone at 4 °C for 5 min. After 3 washing steps, fixed cells were incubated with the primary antibodies for 1 h in a humidified room. The detection of $E6_{F47R}$ was performed using a 1:100-dilution of AbE6P/Gi, whereas for $E7_{GGG}$, a 1:100-dilution of a mixture of ED17/8C9 monoclonal antibodies was used or, alternatively, the FPwt-immunoadsorbed rabbit anti-E7 specific polyclonal antibody, produced in our laboratory. The exceeding antibodies were removed by 3 washing steps, and a 1:100-dilution of the Fluorescein IsoThioCyanate (FITC)-goat anti-mouse or anti-rabbit antibodies (Dako Cytomation). After incubation and 3 washing steps, and a final-one in distilled water, the cover glasses were laid on a drop of a maintaining solution (80% glycerol, 3% N-propyl-gallate in 20% PBS^+, pH 7.4). FPwt-infected cells were used as a negative control, whereas FPE7-infected Vero cells were used as a positive control to

detect the $E7_{GGG}$ protein. The samples were viewed under a Zeiss Axioskop fluorescence microscope.

Production of E6 and E7 proteins

The E6 and E7 recombinant proteins, bound to an Histidine tag were produced using E. Coli JM109 bacteria, containing the pQE30-E6-His or pQE30-E7-His plasmid, following the manufacturer's specifications (Qiagen), with minor modifications. Briefly, bacteria were grown in 50 mL of LB supplemented with 100 µg/mL ampicillin at 37 °C o/n. The culture was then added of 450 mL of fresh medium at 37 °C for 1 h until the optical density of 0.6-0.7 was reached, as measured by a spectrophotometer at 600 nm. The protein production was induced by 2 mM IPTG at 37 °C for 3 h, and, after centrifugation at 4,000 x g at 4 °C for 30 min, the bacterial pellet for the E6 protein was resuspended in buffer A (HCl 6 M Guanidine, 0.1 M NaH_2PO_4 x H_2O, 10 mM Tris HCl, pH 8), and the pellet for the E7 protein in buffer B (100 mM NaH_2PO_4 x H_2O, 10 mM Tris HCl, 8 M Urea, pH 8) before freezing at -80 °C o/n. The pellet was then 1:2-diluted with a lysis solution (0.3 M NaCl, 1% Triton-X-100 in buffer A or B), and incubated at room temperature for 30 min on an orbital shaker. The solution was then sonicated for 1 h, and the lysate was centrifuged at 14,000 x g at 4 °C for 30 min. The supernatant was added of 1% Triton-X-100 and imidazole 20 mM pH 8, and incubated at room temperature for 30 min with a Ni-NTA Agarose resin (Qiagen), previously resuspended in buffer A or B. The resin, bound to the protein, was centrifuged at 800 x g at 4 °C for 5 min, washed once with 1% Triton-X-100, diluted in buffer A or B, and twice with buffer C (100 mM NaH_2PO_4 x H_2O, 10 mM Tris HCl, 8 M Urea, pH 6.3), and then loaded on a column. The protein was eluted from the resin with different fractions of 1 M imidazole, pH 8, and subsequently analyzed by SDS-PAGE to detect which ones

contained the protein of interest. The positive fractions were mixed together and dialyzed against PBS^- to remove the imidazole.

The protein quantification was performed in 96-well plates after preparing standard samples with a known concentration of Bovine Serum Albumin (BSA) to the aim at generating a calibration standard curve from 0.2 to 20 mg/mL. Four wells were loaded with 2 µL/each of protein solution in 20 µL water. After adding 200 µL/well of reaction mixture (Pierce, Rockford, IL, USA), and incubating at 37 °C for 30 min, measurements at 570 nm were performed using a spectrophotometer.

Immunization protocol and challenge with TC-1* tumor cells

Two protocols were followed for each mutated protein using six-week-old C57Bl/6 female mice (Charles River Laboratories, Como, Italy). First is referred to as the immune response protocol, in which the mice were bled before each immunization to evaluate the induced humoral response by ELISA. These mice were finally sacrificed to obtain splenocytes for the enzyme-linked immunospot (ELISPOT) and determine the cellular response. The second regimen is referred to as the therapeutic immunization protocol, in which the mice were inoculated with E6/E7-expressing TC-1* tumor cells before administration of the immunogens. The tumor volume and growth was monitored during the whole experiment.

Immune response protocols

In the immune response protocol, the immunizations were performed to evaluate the response induced by $E6_{F47R}$-based immunogens (Fig. 15 A). Mice were divided in three

groups of six animals each, and they were primed with the recombinant pDNAE6$_{F47R}$ plasmid once (protocol GA.1; 100 µg/mouse, intramuscular, i.m.) or twice (protocol GA.2; 100 µg/mouse, i.m.). Two weeks after the DNA inoculation, the animals were boosted twice (protocol GA.1) or once (protocol GA.2) with FPE6$_{F47R}$ (10^7 PFU/mouse, subcutaneous, s.c.). All of the mice remained in good health, and, after the last immunization, the mice of each group were sacrificed to obtain splenocytes. The control mice (protocol GA.3) were repeatedly mock-infected.

In the E7$_{GGG}$ immune response protocol (Fig. 15 B), mice were divided into three groups of eight animals each. The mice were primed with the recombinant DNAE7$_{GGG}$ plasmid (protocol GB.2; 100 µg/mouse, i.m.) or with the recombinant FPE7$_{GGG}$ virus (protocol GB.3; 10^7 PFU/mouse, s.c.), and boosted three times at 2-week intervals with FPE7$_{GGG}$ virus (protocol GB.2 and GB.3; 10^7 PFU/mouse, s.c.) one week after the first inoculation. The mice for protocol GB.1 were mock-vaccinated and used as controls. After the last immunization, the mice were still in good health, and they were sacrificed to perform the ELISPOT assays.

Therapeutic immunization protocols

In the therapeutic protocol, the immunizations were performed to evaluate the therapeutic efficacy of E6$_{F47R}$-based immunogens (Fig. 15 C), and four groups of five mice were inoculated by s.c. injection of 5×10^3 TC-1* cells in a final volume of 200 µL saline solution. TC-1* were previously found to induce tumors in 100% of the mice [60]. After three and ten days, mice were immunized twice with DNAE6$_{F47R}$ (protocol GC.1; 100 µg/mouse, i.m.) or with DNAE6$_{F47R}$ followed by FPE6$_{F47R}$ (protocol GC.2; 10^7 PFU/mouse, s.c.) or twice with FPE6$_{F47R}$ (protocol GC.3; 10^7 PFU/mouse, s.c.). The mice for protocol GC.4 were mock vaccinated and used as controls.

In the therapeutic protocol for $E7_{GGG}$ (Fig. 15 D), three groups of five or six mice were inoculated by s.c. injection of 5×10^3 TC-1* cells in a final volume of 200 µL saline solution. After three days, the mice were primed once with either $DNAE7_{GGG}$ (protocol GD.2; 100 µg/mouse, i.m.) or with $FPE7_{GGG}$ (protocol GD.3; 10^7 PFU/mouse, s.c.). Boosting was performed one week after the priming, using $FPE7_{GGG}$ (protocol GD.2 and GD.3; 10^7 PFU/mouse, s.c.). The mice for protocol GD.1 were mock vaccinated and used as controls.

In both the therapeutic protocols, tumor growth was monitored after immunization by visual inspection and palpation twice a week. Tumor size was measured with calipers starting from 3 to 4 weeks post challenge (p.c.), and the volume estimated by the formula (width × length × (width + length)/2). Animals were scored as tumor-bearing when tumors reached a size of approximately 1 to 2 mm in diameter and were euthanized for ethical reasons when tumors reached a 2-cm^3 volume. All of the mice were housed and handled under specific pathogen-free conditions, in accordance with the European guidelines for the protection of laboratory experimental animals and laboratory animal care.

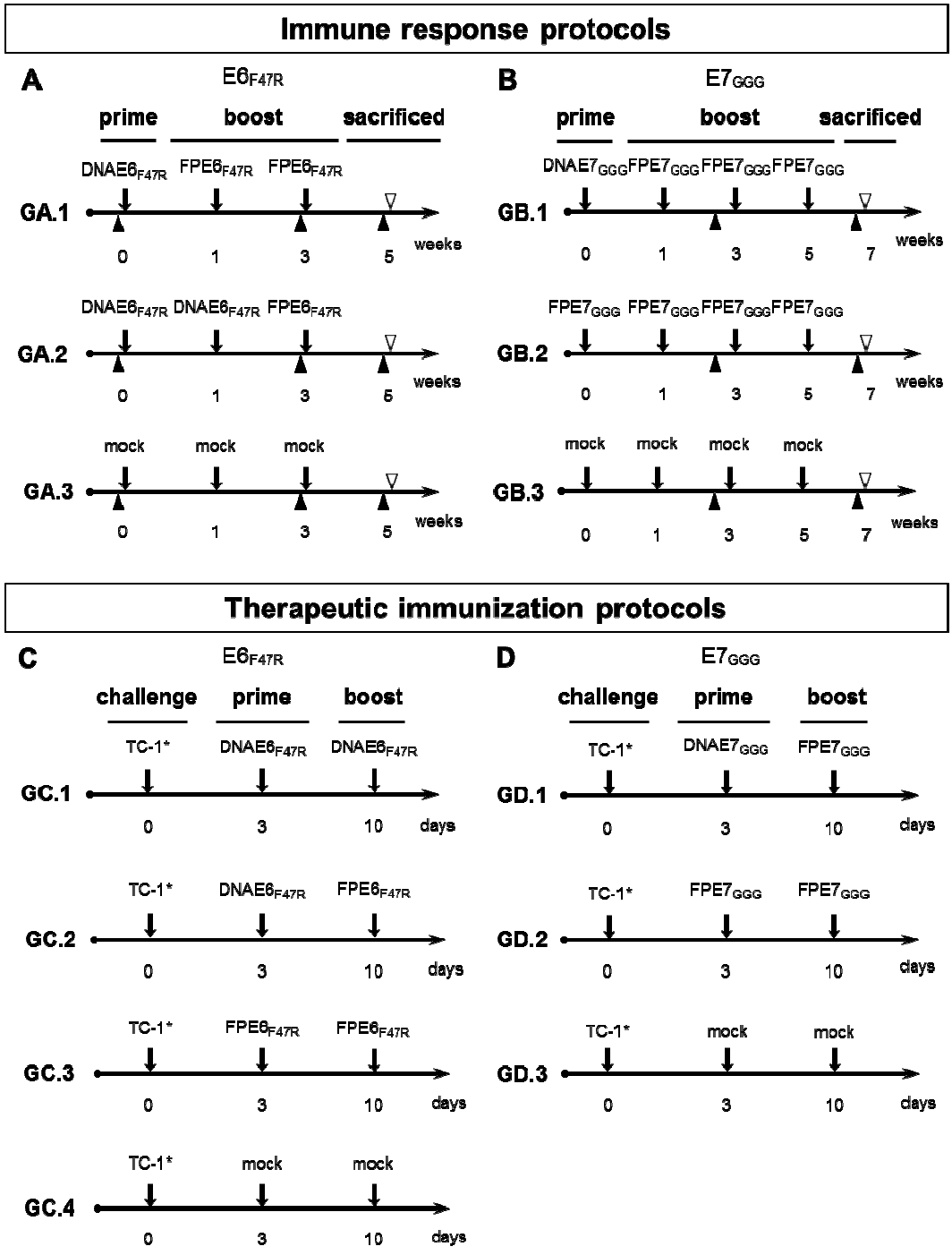

Fig. 15. Immunization protocols

In the immune response protocols, mice were first primed and boosted at one- or two-week intervals, with plasmid or viral recombinants expressing the $E6_{F47R}$ (A) or $E7_{GGG}$ (B) gene.

In the therapeutic protocols, mice were challenged with TC-1* cells, primed once three days after challenge, and boosted one week after the priming, using plasmid or viral recombinants expressing the $E6_{F47R}$ (C) or $E7_{GGG}$ (D) gene.
Bleedings are indicated as black triangles, whereas spleen removal for ELISPOT assays as empty triangles.

ELISA

The mice sera were tested for the presence of antibodies against the E6 and E7 proteins before the first and after each immunization. The purified native pE6 or pE7 proteins (300 ng/well), or the CaSki cells lysates (10^5 cells/well), were adsorbed onto 96-well MaxiSorp microtitre plates (Nunc, Naperville, IL, USA) incubated at 4 °C o/n in a 100-µL Coating Buffer (CB, 15 nM Na_2CO_3, 35 nM $NaHCO_3$, 0.2% NaN_3, pH 9.6) or PBS^-. After the incubation, wells were washed six times with Wash Buffer (WB, 0.8% NaCl, 1.5 nM KH_2PO_4, 2.7 nM KCl, 8.0 nM Na_2HPO_4 (xH_2O), 0.05% Tween-20) saturated with 3% skimmed milk in PBS^-, incubated at 37 °C for 1 h and washed again. Each well was added of 100 µL of sera, 1:50- or 1:100-diluted in WB containing 0.3% skimmed milk, and samples were incubated at 37 °C for 1 h. The plates were then washed 6 times, added of 100 µL of 1:2,000-diluted goat anti-mouse HRP-conjugated antibodies (Dako), and left at 37 °C for 1.5 h before washing again as before. 100 µL of tetra-methyl-benzidine (TMB) substrate (Sigma) were then added and the plate was incubated at room temperature for 30 min in a dark room. The reaction was blocked with 25 µL/well of 2 M H_2SO_4, and the absorbance was measured at 450 nm using a 550 microplate reader (Bio-Rad Laboratories).

ELISPOT assay for IFN-γ-secreting cells

HPV16 E6- and E7-specific T-cell precursors were detected by the ELISPOT assays for IFN-γ-secreting cells (BD^{TM} ELISPOT, BD Biosciences Pharmingen) [120]. Mice were euthanized by cervical dislocation one week after the last immunization and splenocytes were harvested. Briefly, after removal of the exceeding adipose tissue, spleens were mashed, passed through a 100-µm cell strainer, and splenocytes harvested in PBS^{-} before centrifuging at 300 x g at 4 °C for 5 min. Cell pellet was resuspended in RPMI medium (Gibco) supplemented of 10% FCS and P/S (R10) and a single-cell suspension of splenocytes from each group was added to microtiter wells (10^6 cells/well in 100 µL of R10) that had been pre-coated with an anti-mouse-IFN-γ antibody (5 µg/mL; BD Biosciences Pharmingen) along with IL-2 (50 units/mL; Sigma) at 4 °C o/n. The pre-coating was performed by diluting capture antibodies and IL-2 in a final volume of 100 µL/well of Coating Buffer (PBS^{-}) and the plate incubated at 4 °C o/n. The plates were then washed with R10 and incubated at room temperature for 2 h with 100 µL/well of R10. After discarding R10, each well was added a stimulation antigen in a final volume of 100 µL/well of R10, and the splenocytes. The stimulation to detect the T-cell precursor induced by $E6_{F47R}$ immunization was performed using either pE6 in three different concentrations (0.1, 1.0, 10 µg/mL) or two E6-specific epitopes (aa 50-57, aa 18-26) [121,122]. Stimulation to detect T-cell precursors induced by the $E7_{GGG}$ immunization was performed using two different concentrations (1 µg/mL and 10 µg/mL) of either the E7-specific H2Db CTL MHC class I epitope (RAHYNIVTF, aa 49-57) [123] to detect CD8+ T-cell precursors, or the MHC class II peptide (aa 30–67) to detect CD4+ T-cell precursors [124,61]. Stimulations were performed in triplicates at 37 °C for 24-72 h. An unrelated protein (scFv) was used as a negative control. After incubation, cell suspensions were removed, and each well was

washed twice with deionized water and three times with Wash Buffer (0.05% Tween-20 in PBS^-). The plates were incubated with a biotinylated anti-mouse IFN-γ antibody in a final volume of 100 µL/well (2 µg/mL; BD Biosciences Pharmingen) at room temperature for 4 h. After removal of detection antibodies, wells were washed three times with PBS^-, added of 100 µL/well of Streptavidin-HRP and incubated at room temperature for 1 h. Streptavidin-HRP solution was discarded and wells were washed as previously described, before adding 100 µL/well of the 3-amino-9-ethylcarbazole (AEC) substrate and monitoring the spot development in the next 1 to 5 min. The reaction was stopped by washing with deionized water, and, after air-drying in the dark, the spots were counted under a dissecting microscope.

RESULTS

Cloning of the $E6_{F47R}$ sequence into the recombination pFP vector

To the aim at cloning the $E6_{F47R}$ gene into the pFP recombination plasmid, three different strategies were followed.

The $E6_{F47R}$ was first amplified by PCR from the pSG5-$E6_{F47R}$ plasmid, using primers which allowed the generation, at the ends of the gene, of two restriction sequences compatible with the ones contained into the multicloning site of the pcDNA3 intermediate and the final pFP recombination vector. During these steps, both the sub-cloning of the exogenous gene into the intermediate vector and the direct cloning into pFP failed in that all of the obtained clones did not contain the correct plasmid and two alternative strategies were pursued.

First, the $E6_{F47R}$ gene was excised from the starting plasmid pSG5-$E6_{F47R}$, ligated to an intermediate vector, and excised again, to be inserted into the pFP recombination vector. By this strategy, some clones were isolated that contained the insert in the correct orientation. The electrophoretic separation, performed after enzymatic digestion of the intermediate sub-cloning vector, showed the presence of a 751-bp specific band in 13 clones out of the 16 examined (Fig 16, Panel A, lanes 1-4, 6-12, 14-15), whereas when performed on the final pFP vector, 9 out of 14 colonies showed the presence of a 956-bp specific band (Fig 16, Panel B, lanes 1, 3-7, 10, 12-13).

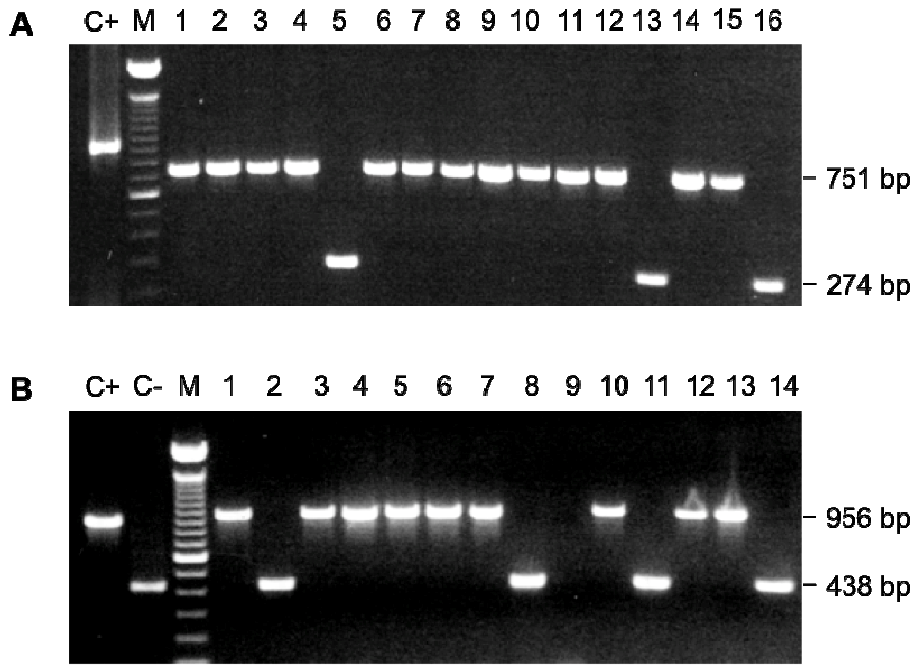

Fig. 16. Analysis of the presence of the $E6_{F47R}$ insert by gel electrophoresis (second strategy)

A. PCR analysis of $E6_{F47R}$ clones in the intermediate pcDNA3 vector: 13 positive clones were positive out of 16.
B) PCR analysis of $E6_{F47R}$ clones in the recombination pFP vector: 9 positive clones were positive out of 14

M, molecular weight marker, 100 bp
C+, positive control (pcDNA3 containing an exogenous gene (A) and pFPE6 (B)
C-, negative control (empty pFP)

Following an alternative cloning strategy, the $E6_{F47R}$ gene was amplified by PCR and directly used, without purification, to ligate the blunt ends to the intermediate pCR-BluntII-TOPO vector. The correct 720 bp- band was identified in 29 clones out of 30 (Fig. 17, Panel A lanes 1-8 e 10-30).

Six clones were chosen and further analyzed by two different enzymatic digestions. The first, using EcoRI, showed two bands of 501 and 3,932 bp, confirmed the presence of the insert (Fig. 17, Panel B, lanes 1-6 EcoRI), whereas the second, performed with HindIII, showed two bands of 477 and 3,956 bp, respectively, demonstrated not only the presence of the insert, but also the maintenance of the restriction sequences flanking the $E6_{F47R}$ gene, and necessary for the subsequent cloning steps (Fig. 17, Panel B, lanes 1-6 HindIII). The $E6_{F47R}$ gene was then excised from one of the selected clones by cutting with the HindIII, and inserted into pFP, which was previously cut with the same enzyme. The presence of the recombinant gene, verified by PCR, highlighted a 956-bp band in 7 clones out of 19 (Fig. 17, Panel C, lanes 1-3, 7-9, 11-13, 15-18). Two different enzymatic digestions confirmed the presence of the exogenous gene in all of these clones. In particular, HindIII digestion showed in each selected clone, a band of 8,209 bp corresponding to the vector, and the specific 477-bp band of the insert (Fig. 17, Panel D, lanes 1-7 HindIII). Conversely, the SalI digestion, showed the presence of the $E6_{F47R}$ gene in the right orientation only in 2 clones (Fig. 17, Panel D, lanes 4-5 SalI) by the presence of a 417-bp and a 8,269-bp bands. Both the positive clones were sequenced and no mutations in were evidenced.

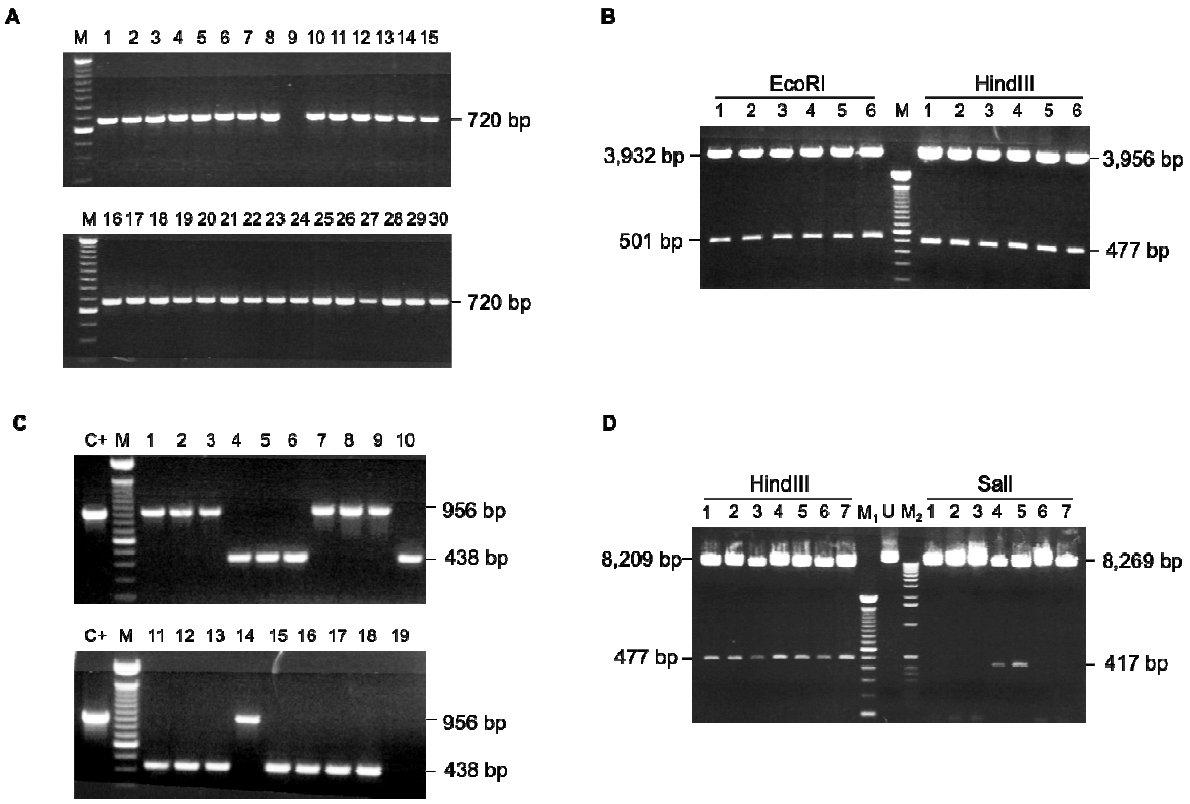

Fig. 17. Analysis of the presence of $E6_{F47R}$ transgene by gel electrophoresis (third strategy)

PCR analysis of $E6_{F47R}$ clones in the intermediate pCR-BluntII-TOPO vector. (A) 29 positive clones were positive out of 30. (B) The presence of the transgene was confirmed by enzymatic digestion with EcoRI and HindIII.

PCR analysis of $E6_{F47R}$ clones in the recombination pFP vector. (C) 7 positive clones were positive out of 19 clones. (D) The presence of the transgene and its correct orientation was confirmed by enzymatic digestion with HindIII and SalI.

M o M1, molecular weight marker, 100 bp
M2, molecular weight marker, 1 kb
C+, positive control (pFPE6)
U, uncut plasmid

Cloning of the $E7_{GGG}$ sequence into the recombination pFP vector

The cloning of $E7_{GGG}$ gene into the pFP recombination vector was performed after PCR amplification of the transgene, cut by restriction enzymes and its direct insertion into the vector. To the aim at evaluating the correct cloning of the insert, a screening was performed by PCR using a primer complementary to the VVH6 promoter sequence, present on the vector, and a primer complementary to the transgene. This allowed the visualization of positive clones and of the correct orientation of them. After running in agarose gel, the expected 400-bp sequence was shown in 8 out of 44 clones (Fig. 18, lanes 3, 12, 25-27, 38, 43-44). Sequencing was also performed, which confirmed the absence of mutations into the whole sequence.

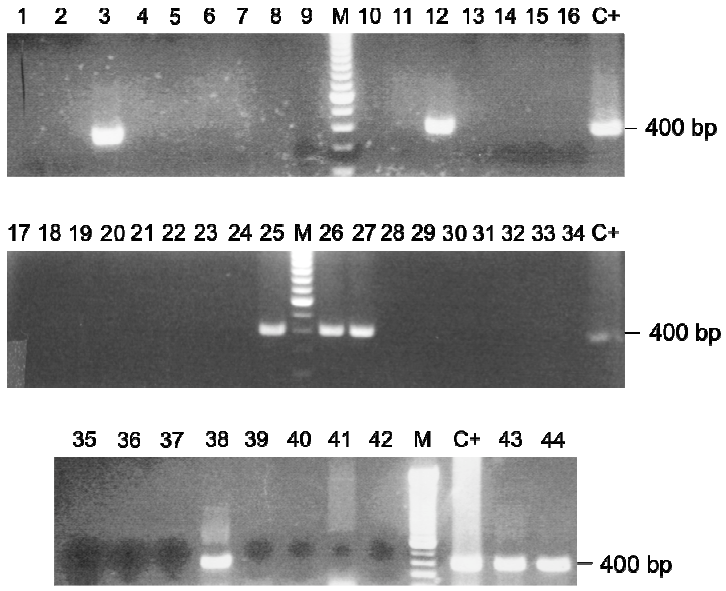

Fig. 18. Analysis of the presence of the $E7_{GGG}$ insert by gel electrophoresis

PCR analysis of $E7_{GGG}$ clones in the recombination pFP vector: 8 positive clones were positive out of 44.

M, molecular weight marker, 100 bp
C+, positive control (pFPE7)

Construction and selection of the viral recombinants

The construction of the $FPE6_{F47R}$ and $FPE7_{GGG}$ viral recombinants was performed by IVR, whereas the selection of clones containing the heterologous gene was achieved by hybridization with a radiolabeled probe and the autoradiography (Fig. 19). Since the frequency of recombination between the FPwt virus and the recombination plasmid is very low, to the aim at obtaining a pure viral recombinant stock, plaque purification was performed by subsequent and repeated steps, to obtain a complete separation of the recombinant clones from the FPwt viruses. Viral clones were considered pure when the numbers of radiography-positive plaque were coincident with the plaque numbers originally present on the monolayer of infected cells.

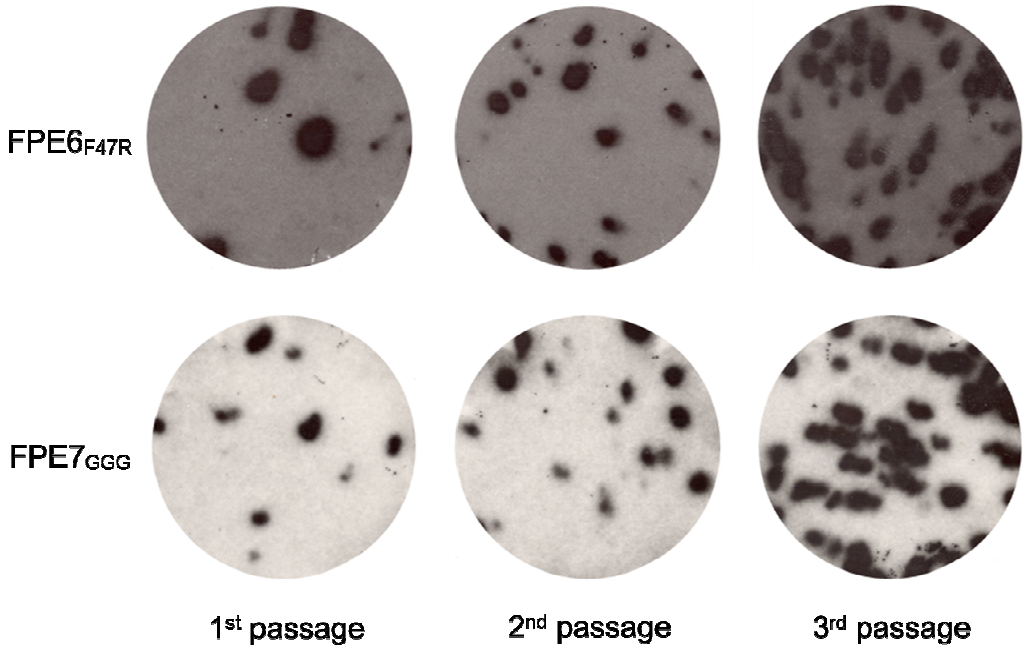

Fig. 19. Plaque purification after *in-vitro* recombination

Nitrocellulose membranes were hybridized with the radiolabeled probe. The different purification passages showing the increase of positive plaque numbers.

Different clones for each recombinant were amplified, titered and then analyzed to verify their ability to express the heterologous gene. The analyses were performed by different assays, starting from RT-PCR and then by Western blotting and immunofluorescence.

FPE6$_{F47R}$ and FPE7$_{GGG}$ recombinants express the E6$_{F47R}$ and E7$_{GGG}$ mRNAs

The transgene expression driven by the viral recombinants bearing E6$_{F47R}$ or E7$_{GGG}$ was first assessed by RT-PCR, performed on mRNA samples extracted from CEF, Vero and MRC-5 cells, previously infected with FPE6$_{F47R}$ or FPE7$_{GGG}$.

Agarose gel separation of the sequences amplified by RT-PCR highlighted the presence of specific 477-bp bands in samples infected with FPE6$_{F47R}$ (Fig. 20, Panel A), or 298-bp bands in samples infected with FPE7$_{GGG}$ (Fig. 20, Panel B). These bands were similar to those obtained in samples transfected with the expression plasmids, used as positive controls (C+). In particular, FPE6$_{F47R}$ showed a higher transcript level in human MRC-5 cells than in permissive CEF cells or non-permissive Vero cells (Fig. 20, Panel A, lanes b). The intensity of the bands in FPE7$_{GGG}$-infected samples was slightly higher in mammalian cells than in CEF cells (Fig. 20, Panel B, lanes d). No specific bands were amplified in mock-infected (Fig. 20, Panel B, lanes c) or FPwt-infected cells (Fig. 20, Panels A and B, lanes a), used as negative controls.

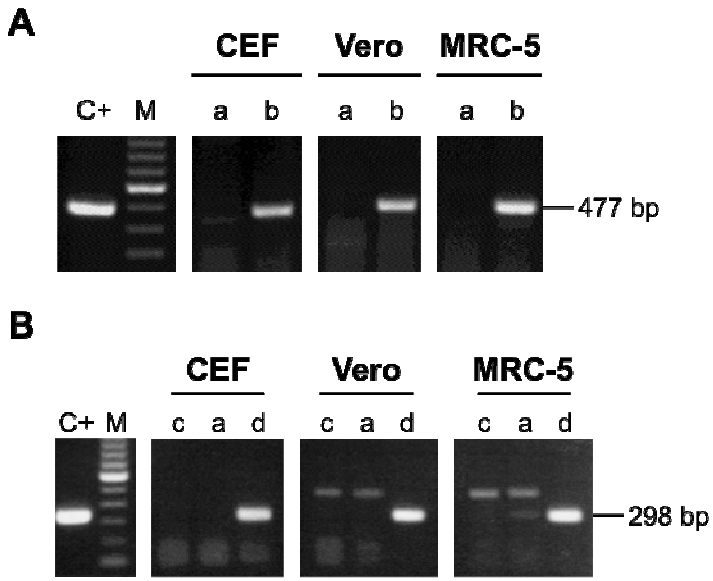

Fig. 20. Analysis of the transcript expression by FPE6$_{F47R}$ and FPE7$_{GGG}$ recombinants

RT-PCR amplification of E6$_{F47R}$ (A) and E7$_{GGG}$ (B) transcripts from cell lysates derived from different cell lines, infected with the viral recombinants.

Mock-infected cells (lanes a); FPwt-infected cells (lanes c); FPE6$_{F47R}$-infected cells (lanes b); FPE7$_{GGG}$-infected cells (lanes d)
M, molecular weight marker, 100 bp
C+, positive control (pFPE6$_{F47R}$ (A) and pFPE7$_{GGG}$ (B))

FPE6$_{F47R}$ and FPE7$_{GGG}$ recombinants express the respective heterologous proteins

The correct expression of the heterologous E6$_{F47R}$ and E7$_{GGG}$ proteins was verified by Western blotting assays performed on cell lysates obtained from cells infected with the viral recombinants.

The specific anti-E6 primary antibody, AbE6/Mu, allowed to detect a 19-kDa band representing the E6$_{F47R}$ protein (Fig. 21, Panel A, lanes 2, 4 and 6). The non-mutated E6 protein (pE6), produced by an engineered bacterial vector, was used as a molecular weight control marker (Fig. 21, Panel A, lane 7). The E6$_{F47R}$ protein was expressed at higher levels by CEFs (5.8-fold) and Vero cells (4.4-fold) than by MRC-5 cells, as determined by densitometric analyses.

Western blotting of FPE7$_{GGG}$-infected cell lysates showed the presence of an 18-kDa band representing the mutated E7 oncoprotein (Fig. 21, Panel B, lanes 3 and 6). The non-mutated E7 protein (pE7) produced by an engineered bacterial vector (Fig. 21, Panel B, lane 7) was used as a molecular weight control marker. CaSki cells (Fig. 21, Panel B, lane 8) and CEFs and Vero cells infected with FPE7 were used as positive controls for the E7 detection (Fig. 21, Panel B, lanes 1 and 4). It is worth noting that different monoclonal antibodies were used to determine the expression of these HPV proteins, and only some of them showed specificity and an adequate avidity in the different tests. In particular, the 8C9 monoclonal antibody (Zymed) could not recognize the E7 protein produced by the FPE7$_{GGG}$ recombinant, which, conversely, was recognized by the ED17 monoclonal antibody (Santa Cruz Biotechnology), whereas a mixture of both (1:1) provided the best results.

FPE7 was always expressed at higher levels, when compared to $FPE7_{GGG}$ for the same cells, and the amount of $E7_{GGG}$ was 2.5-fold higher in Vero cells than in CEFs, as determined by densitometric analyses. No specific bands were seen when cells were infected with FPwt (Fig. 21, Panel A, lanes 1, 3 and 5; Panel B, lanes 2 and 5).

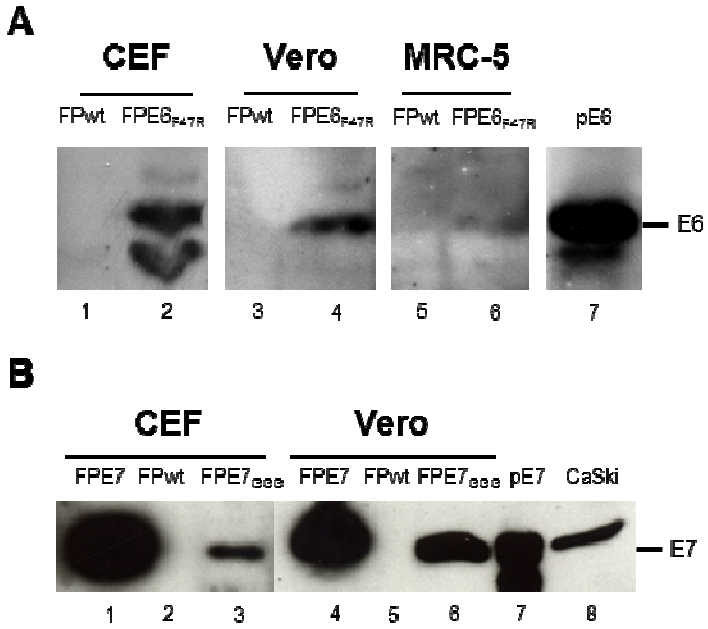

Fig. 21. Western blot analysis of the transgene expression by $FPE6_{F47R}$ and $FPE7_{GGG}$ recombinants

The heterologous gene expression was determined by identifying the $E6_{F47R}$ (A) or $E7_{GGG}$ (B) exogenous proteins on different cell lysates of $FPE6_{F47R}$- or $FPE7_{GGG}$-infected cells

A)	lanes 1, 3 and 5	FPwt-infected cells
	lanes 2, 4 and 6	$FPE6_{F47R}$-infected cells
	lane 7	E6 protein, C+
B)	lanes 2 and 5	FPwt-infected cells
	lanes 3 and 6	$FPE7_{GGG}$-infected cells
	lanes 1 and 4	FPE7-infected cells, C+
	lane 7	E7 protein, C+
	lane 8	CaSki cells, C+

To verify the localization of the heterologous protein expressed by the two FP recombinants, immunofluorescence assays were carried out on CEFs, Vero and MRC-5 cells depending on the viral recombinant.

After $FPE6_{F47R}$-infection, a granular fluorescence was detectable in CEFs and Vero and MRC-5 cells (Fig. 22, Panel A, 1b, 2b, 3b) using the AbE6/Gi mouse polyclonal antibody, with nuclear and perinuclear/cytoplasmic localization. No particular differences were seen in the different cell lines used during the assays. In fact, although Vero cells seem to be more fluorescent than other cell lines, they present a higher background fluorescence when infected with the empty vector.

After $FPE7_{GGG}$-infection, immunofluorescence was detectable only in Vero cells (Fig. 22, Panel B, 1b) using the rabbit polyclonal antibody, with nuclear/perinuclear/cytoplasmic localization and granular distribution.

The FPE7-infected cells (Fig. 22, Panel B, 1c) used as a positive control showed a higher positivity, but no differences were seen in the cellular localization, although the fluorescence was more granular than in $FPE7_{GGG}$-infected samples.

FPwt-infected cells were always negative (Fig. 22; Panel A, 1a, 2a and 3a; Panel B, 1a), as expected.

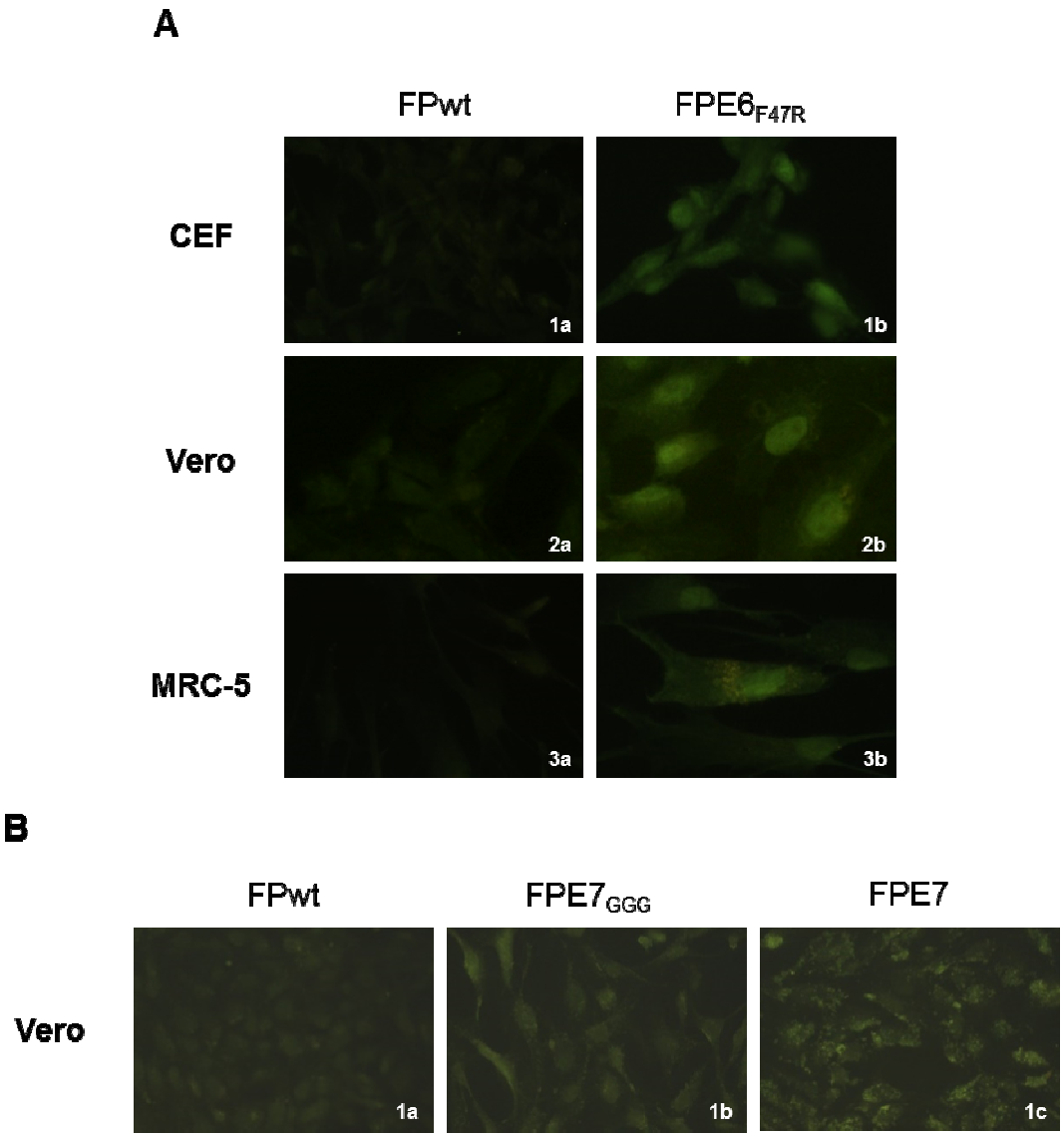

Fig. 22. Analysis of the $E6_{F47R}$ (A) and $E7_{GGG}$ (B) proteins' localization

The expression and cellular localization of the heterologous $E6_{F47R}$ and $E7_{GGG}$ proteins were assessed by immunofluorescence performed on $FPE6_{F47R}$- or $FPE7_{GGG}$-infected cells (A and B, respectively).

A) Panels 1a, 2a and 3a FPwt-infected cells, C-
Panels 1b, 2b and 3b $FPE6_{F47R}$-infected cells

B) Panel 1a FPwt-infected cells, C-
Panel 1b $FPE7_{GGG}$-infected cells
Panel 1c FPE7-infected cells, C+

The E6- and E7-specific humoral responses are very low in vaccinated mice

The immunized mice were tested for E6- and E7-specific humoral immunity to evaluate the ability of the different vaccine regimens to stimulate the production of antibodies and their capacity to recognize the HPV naïve oncogenic E6 and E7 proteins.

ELISA, performed in plates coated with either the native purified recombinant pE6 protein or the CaSki cell lysates, allowed to measure the antibody response against E6. In the immune response protocol, the mice sera were collected before the first immunization (T0), and after the first and the last boosts (T2 and T3, respectively). The antibody responses against E6 were generally low (Fig. 23, Panel A), but they increased after the third immunization, although this effect was seen only when the assay was performed after plating CaSki cell lysates. In particular, a more evident increase occurred after the last boost in mice immunized twice with $DNAE6_{F47R}$ followed by a $FPE6_{F47R}$ boost (group GA.2).

As for $E7_{GGG}$, ELISA, using either plates coated with the purified recombinant pE7 protein or CaSki lysates, was performed to measure the specific anti-E7 antibody response in vaccinated mice. The sera of the immunized mice were collected after the first (T1) and third (T3) boosts and analyzed. Again, the antibody responses were generally low (Fig. 23, Panel B), but they increased after the third immunization when the assays were performed plating either the E7 protein or CaSki cell lysates. In particular, mice immunized only with the $FPE7_{GGG}$ (group GB.2) showed a higher antibody level after the last immunization, both when using pE7 or CaSki cell lysates, whereas mice that received the $DNAE7_{GGG}$ prime followed by $FPE7_{GGG}$ boosts (group GB.1) showed at T3 an increase, which was more evident when ELISA was performed using the E7 protein. The anti-E7 antibody

levels of mock-infected mice were lower when evaluated on the purified protein, whereas they showed an increase when assessed on CaSki cell lysates.

Experiments using carbonate buffer or PBS as a coating buffer showed similar results (data not shown).

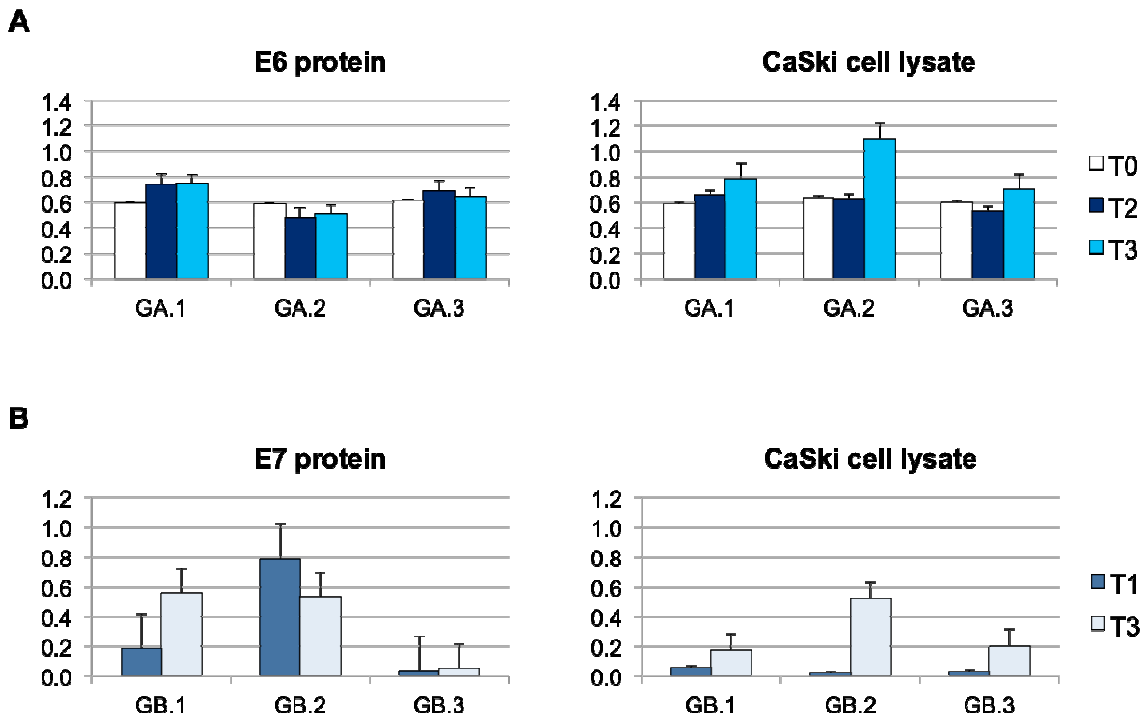

Fig. 23. Analysis of the anti-E6 and anti-E7 humoral responses

The identification of the antibody responses against the E6 or E7 proteins induced by the two immune response protocols were assessed by ELISA after plating either the purified proteins or CaSki cell lysates.
Data are expressed as means for each group of mice ± standard error. T0, T1, T2 and T3 are the time of serum collection before or after mice immunization, depending on the vaccine protocol.

A $E6_{F47R}$
GA.1 DNA/FP/FP
GA.2 DNA/DNA/FP
GA.3 mock/mock/mock

B $E7_{GGG}$
GB.1 DNA/FP/FP/FP
GB.2 FP/FP/FP/FP
GB.3 mock/mock/mock/mock

Specific cell-mediated responses are observed in immunized *vs* mock-vaccinated mice

IFN-γ-secreting spot-forming cells (SFCs) were counted using splenocytes obtained after the last immunization of the immune response protocols. The analyses were performed with the aim at evaluating the ability of the immunization protocols to induce specific cell-mediated immune responses. Data are presented as fold increases related to the responses obtained in cells stimulated with an unrelated protein.

In animals immunized with the recombinants expressing the $E6_{F47R}$ gene, an increase in IFN-γ-producing cells was seen for both groups of vaccinated mice, above the level observed in the mock-vaccinated mice of protocol GA.3 (Fig. 24, Panel A). In particular, when the splenocytes were stimulated with the E6 protein, a significantly increased response was shown by protocol GA.1 (3.4-fold increase, $p < 0.001$) and protocol GA.2 (3.2-fold, $p < 0.001$) *vs* GA.3. When the E6-specific aa 50-57 peptide was used, this increase was 6-fold higher for GA.1 ($p < 0.001$) and 5.1-fold higher for GA.2 ($p < 0.01$) than in GA.3. When the aa 18-26 peptide was used, a 11.5-fold increase for GA.1 ($p < 0.001$) and 4.0-fold increase for GA.2 ($p < 0.05$) *vs* GA.3 were observed. Statistical differences between the GA.1 and GA.2 vaccinated groups were seen only when the test was performed by stimulating cells with the aa 18-26 E6 peptide, showing a 2.9-fold increased response in GA.1 protocol ($p<0.001$) *vs* GA.2.

Mice immunized with different recombinants expressing the $E7_{GGG}$ gene showed an increase in IFN-γ-producing cells in both GB.1 and GB.2 groups, above the level observed in the mock-vaccinated mice of group GB.3 (Fig. 24, Panel B). In particular, when splenocytes were stimulated with the E7-specific aa 49-57 peptide, which is associated to

the induction of CD8 precursors, protocols GB.1 and GB.2 showed a 15.6-fold ($p<0.001$) and 3.5-fold ($p<0.05$) increased response vs GB.3. When the E7-specific aa 30-67 peptide was used, which is associated to the induction of CD4 precursors, the increase was of 9.9-fold for GB.1 ($p<0.001$) and 3.6-fold for GB.2 ($p<0.05$) *vs* GB.3. The comparison between the two different immunization protocols (GB.1 *vs* GB.2) highlighted a 4.45-fold ($p<0.001$) increased response when the stimulation was performed with the E7-specific aa 49-57 peptide, and a 2.75-fold ($p<0.001$) increased response when the stimulation was performed with the E7-specific aa 30-67 peptide. Different epitope concentrations for CD4 and CD8 gave similar results (data not shown).

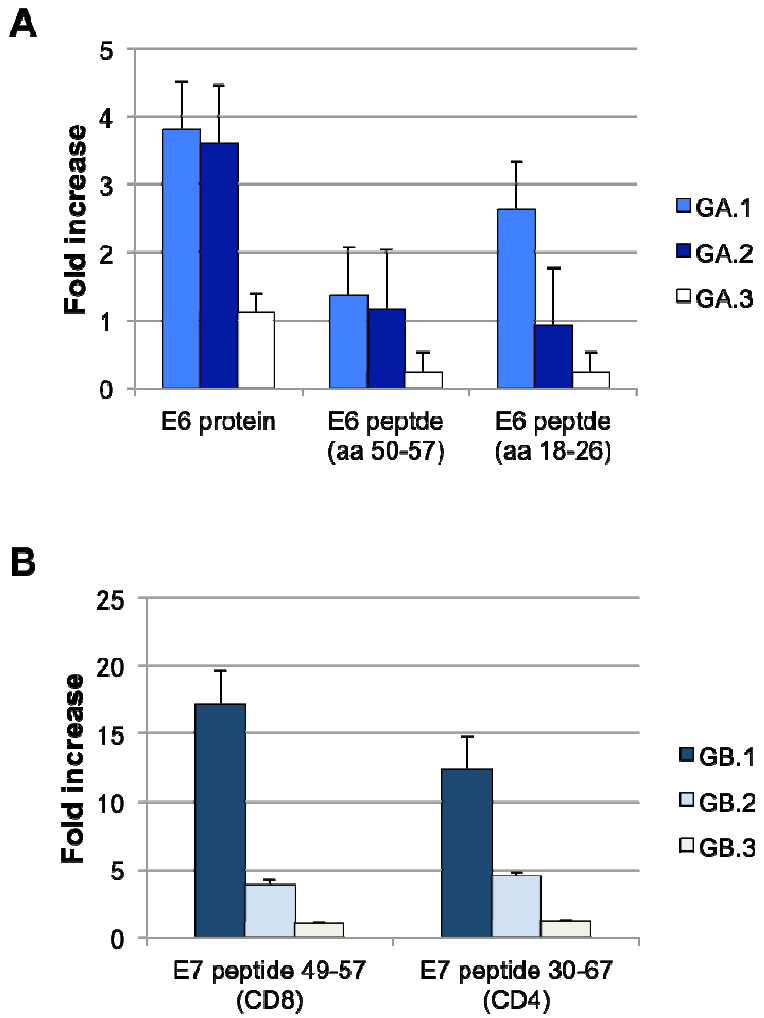

Fig. 24. Functional virus-specific T-cell responses to E6 or E7

IFN-γ production was measured by ELISPOT assays after specific antigenic stimulation with the E6 protein or relative peptides (A) or with E7 peptides (B) to detect the cell-mediated immune responses. The number of IFN-γ-secreting spot-forming cells was assessed after subtracting the number of cells producing IFN-γ in the absence of stimulation. Data are presented as fold-increase responses to the specific protein or peptides, compared to responses to an unrelated protein, and they represent the means of all of the mice for each group ± standard error.

A $E6_{F47R}$ GA.1 DNA/FP/FP
GA.2 DNA/DNA/FP
GA.3 mock/mock/mock

B $E7_{GGG}$ GB.1 DNA/FP/FP/FP
GB.2 FP/FP/FP/FP
GB.3 mock/mock/mock/mock

Therapeutic immunization delays tumor growth

After injecting the TC-1* cells into naïve mice, prime and boost immunizations were performed on days 3 and 10 p.c., respectively.

When immunotherapeutic treatments were performed with recombinants expressing the $E6_{F47R}$ protein, the number of tumor-bearing animals was significantly lower in the immunized mice compared to the control group (Fig. 25, Panel A; GC.1, GC.2, GC.3 *vs* GC.4, $p < 0.001$). In particular, tumor appearance was delayed to day 21 p.c. in 20% of the mice of GC.1 and GC.3, and to day 27 p.c. in 40% of the animals of group GC.2. Tumor development was seen in 80% of the mice at day 17 p.c. in group GC.4, and at day 34 and 31 p.c. in groups GC.2 and GC.3. At day 54, only 20% of the animals of group GC.1 developed tumors, whereas, cancer-bearing mice remained 80% in groups GC.2 and GC.3. The numbers of healthy mice remained higher in group GC.1, when compared to both groups GC.2 and GC.3. Statistical analyses also indicate a significant difference between group GC.2 *vs* GC.1 ($p < 0.05$) and group GC.3 *vs* GC.1 ($p < 0.01$).

Also, mice treated with the different recombinants expressing the $E7_{GGG}$ gene showed a significant difference in the percentage of tumor-bearing animals among the three groups (Fig. 25, Panel B). In particular, a significant difference was found between groups GD.2 *vs* GD.3 and GD.1 *vs* GD.2 ($p<0.05$ and $p<0.01$, respectively). Tumor appearance was delayed to day 23 p.c. in 40% and 33.3% of the mice of groups GD.3 and GD.1, respectively, whereas tumors were detectable earlier (day 16 p.c.) in 16.7% of the animals of group GD.2. Tumors developed in around 50% of the mice earlier in group GD.2 (day 21 p.c.) and by day 27 p.c. in groups GD.3 and GD.1. By day 45, only in group GD.3 tumors developed in 100% of the animals, whereas, by the same day, tumor-bearing mice were 50% in group GD.1 and 83.3% in group GD.2.

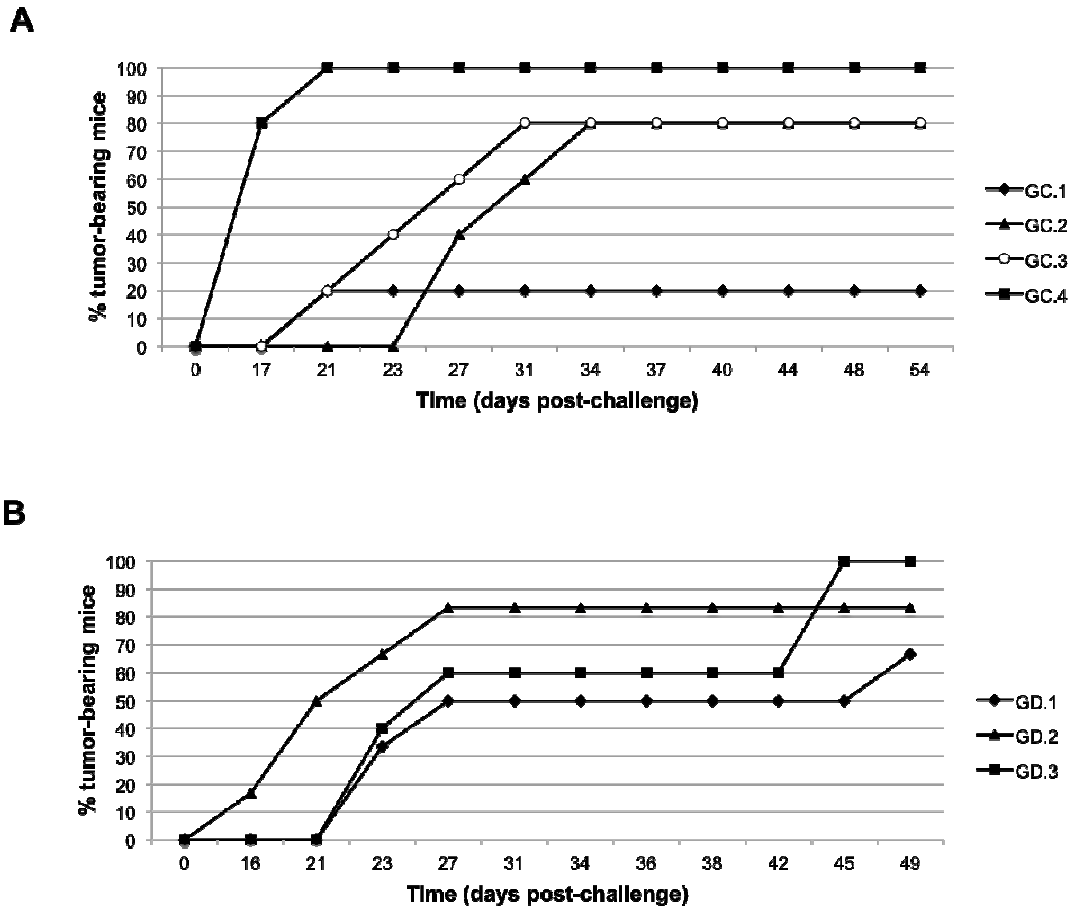

Fig. 25. Tumor growth after the therapeutic immunization

Mice were challenged with TC-1* cells and immunized at days 3 and 10 p.c. with the plasmid or viral immunogens expressing the $E6_{F47R}$ (A) or the $E7_{GGG}$ (B) genes. Tumor development was assessed by palpation and data are presented as the percentage of tumor-bearing mice after challenge.

A $E6_{F47R}$
GC.1 TC-1*/DNA/DNA
GC.2 TC-1*/DNA/ FP
GC.3 TC-1*/FP/FP
GC.4 TC-1*/mock/mock

B $E7_{GGG}$
GD.1 TC-1*/DNA/FP
GD.2 TC-1*/FP/FP
GD.3 TC-1*/mock/mock

DISCUSSION

Prevention and therapy of HPV-related pathologies, such as cervical cancer, can be pursued both by prophylactic and therapeutic vaccines. Currently available vaccines, Gardasil® and Cervarix®, target L1 capsid protein of high risk HPV strains to prevent the viral infection, but they show some limitations due to their high-cost of production, and their restriction to only a few viral genotypes. At present, it is also impossible to determine their protective long-term durability and efficacy.

Present scientific researches aim therefore at developing new low-cost production systems, as well as VLPs containing also the L2 capsid protein, which is more conserved among the different HPVs genotypes and presents a high number of cross-reactive epitopes [125]. Although current vaccines are able to protect healthy subjects from HPV infection, they cannot be effective to previously infected individuals, and thus, they are completely inadequate to treat already established lesions. Considering the high numbers of HPV-infected subjects and the time lag between infection and tumor appearance, the development of an effective therapeutic vaccine is a priority.

These vaccines aim at inducing a complete response, in particular by simulating a cell-mediated immunity against the early-expressed HPV antigens, such as the E6 and E7 proteins, rather than against the capsid antigens. In fact, E6 and E7 proteins are constitutively expressed both in precancerous lesions and in already established HPV-related cancers. New strategies involve the construction of chimeric VLPs containing both the L1 protein in association with E6 and E7 , with the aim at developing preventive and therapeutic vaccines in a single formulation [126].

The aim of this project was the construction of new immunogens expressing genes coding for the mutated non-oncogenic forms of the HPV16 E6 and E7 proteins ($E6_{F47R}$ and $E7_{GGG}$, respectively), the evaluation of their ability to induce a specific immune response in vaccinated mice and their therapeutic efficacy on a mouse model of cervical tumor. Modifications inside the gene sequences make these immunogens safer, since they are devoid of the oncogenic activity of the native protein forms.

The $E6_{F47R}$ gene was cloned into the pFP recombination plasmid following different strategies. Some problems were encountered during the insertion of the heterologous gene into the intermediate and the final avian vectors. In fact, when the $E6_{F47R}$ gene was cut out from the original plasmid using specific restriction enzymes, both the subcloning and the final cloning were achieved easily, whereas, when the gene was amplified by PCR, cut with the appropriate restriction enzymes and purified, the ligation into the recombination plasmid did not occur. Although it is still unclear, we hypothesize that the ligation event might have been hampered by an incomplete digestion or by the purification process performed before the ligation. Although the direct insertion into pcDNA3 and pFP vectors was obtained easily, this strategy generated an exceeding 100-bp region between the promoter and the starting codon of the heterologous gene, which might have impaired the coding process. Thus, a parallel strategy was followed by cloning the $E6_{F47R}$ gene amplified by PCR without performing any enzymatic restriction reaction and purification before inserting the gene into the vector. This hypothesis was confirmed, as the gene was correctly cloned inside the intermediate vector and then into the final pFP recombination plasmid.

Due to the similar size of the modified E6 and E7 genes, it still remains unclear why, following the same strategy of PCR amplification, the insertion problems have raised only

for $E6_{F47R}$ and not for $E7_{GGG}$. Even if the amplification strategy by PCR allows the preparation of more precise constructs, it seems to be less efficient when a subsequent cloning has to be performed. However, it represents a valid strategy, which might be dependent on the restriction enzyme used or on the restriction site in the flanking regions.

To the aim at generating a recombinant vaccine able to induce a specific immune response, and therefore with a preventive and therapeutic efficacy, it is necessary that the heterologous gene inserted into the vector is correctly expressed and presented to the immune system. To this purpose, the expression of the exogenous genes inserted in the $FPE6_{F47R}$ and $FPE7_{GGG}$ viral recombinants was evaluated, by analyzing the presence of the specific transcripts by RT-PCR and then by verifying the synthesis of the corresponding protein by Western blotting and immunofluorescence.

The RNA extracted from CEF, Vero and MRC-5 cells infected with $FPE6_{F47R}$ or $FPE7_{GGG}$ recombinants showed the presence of specific transcripts in all of the cell lines, with a slightly higher levels in human MRC-5 cells. Thus, the insertion of the mutated forms of the heterologous genes do not interfere with their expression in the different cell lines, although it seems that expression is better in human cells, where the viral replication cycle is abortive.

Although the amounts of the $E6_{F47R}$ transcripts expressed by $FPE6_{F47R}$ were similar in all of the cell lines, Western blotting showed a different protein expression level. This cannot be ascribed to a lower efficiency of the $E6_{F47R}$ gene expression by the FP vector, as protein expression was evident in all of the infected cells assessed by immunofluorescence. In CEF, as well as in the positive control, an extra-band was seen, also described in other studies [127,128]. Although this might be due to an already observed splicing process [129], this cannot be the case of FP viruses that replicate in the

cytoplasm. The presence of an additional ATG, downstream the initial starting codon [127], can in fact result in the synthesis of a lower-MW E6 (151 aa, around 18 kDa) in some cell lines, such as in CaSki cells, besides the full length E6 protein (158 aa, around 19 kDa).

Similarly, the amount of $E7_{GGG}$ RNA after $FPE7_{GGG}$ infection was comparable in CEF, Vero and MRC-5 cells, showing only slight differences, whereas by Western blotting a 2.5-fold lower protein expression was seen in CEF, which can be explained by the complete replicative cycle and cytopathic effect of the vector in these cells. A 7.2- and 2.2-fold higher expression was also shown in CEF and Vero cells infected with FPE7 over the mutated $FPE7_{GGG}$ recombinant. This has not been described previously using recombinant plasmids [96] or plant-produced vaccines [130], and it might therefore be ascribed to the lower efficiency of $E7_{GGG}$ gene expression by the FP vector. Unexpectedly, protein detection by Western blotting using a mixture of the anti-E7 ED17 and 8C9 monoclonal antibodies recognized both the native and the mutated proteins, whereas the 8C9 monoclonal antibody alone only recognized the non-denatured protein. Although we do not have an explanation for this discrepancy, if this was due to the disruption of a conformational site by substituting the GLYGYG motif for the DLYCYE sequence [96], the $E7_{GGG}$ recognition by the ED17 monoclonal antibody alone should occur equally also when used alone, and not only when used with 8C9. The difficulty of $E7_{GGG}$ recognition by the monoclonal antibodies was also observed when assessed by immunofluorescence, where, in contrast to what already reported [131], the separate or mixed monoclonal antibodies did not bind the native E7 and mutated $E7_{GGG}$ proteins, which were recognized only by the rabbit polyclonal antibody developed in our laboratory.

The antibody responses against the E6 protein, which were evaluated by ELISA ($E6_{F47R}$ immune response protocol), were detectable only after the third immunization in the

animals following the DNA/DNA/FP protocol (GA.2) and when using the CaSki cell lysates. At present, we have no explanation for the different recognition of the E6 antigen produced in its native form by E. coli or by CaSki cells, although this has already been reported for the responses against E6 and E7 produced in the same way [132]. Conversely, the numbers of IFN-γ-secreting cells were significantly higher after immunization with the $E6_{F47R}$ recombinants, which are essential in clearing TC-1* tumor cells, and can overcome the lack of anti-E6 antibody response. Surprisingly, although the aa 18-26 subdominant epitope may contribute only slightly to the antitumor effect [122], a higher response by the IFN-γ-producing splenocytes was detected to this peptide in the DNA/FP/FP *vs* DNA/DNA/FP (GA.1 vs GA.2) immunization protocol. HPV16 E6 epitope mapping [133] revealed that the aa 50-57 region represents the minimal core sequence essential for E6-specific CTL activity, and that the aa 48-57 peptide is the optimal immunodominant sequence. The E6 aa 50-57 deletion prevented protection of TC-1-challenged mice from a DNA vaccine [121,134], and it is to be noted that the E6 aa 48-57 peptide contains CTL epitopes that are presented by E6-expressing TC-1 cells. Although it has still not been determined which HLA-A24-restricted epitope is the most immunogenic one and the most suitable for immunotherapy, a novel potent HPV16 E6 aa 66-74 peptide has recently been reported, that can be used in vitro as an efficient means of CTL induction [134] to discriminate between the different immunization regimens.

As for the $E6_{F47R}$ protocol, sera collected from mice and immunized with recombinants expressing the $E7_{GGG}$ gene (immune response protocol) showed a low antibody response after ELISA analyses, which only increased slightly over time and might be ascribed to the inadequate antibody affinity and/or avidity. However, the numbers of IFN-γ-secreting cells showed a limited increase after $FPE7_{GGG}$ priming (GB.2 group), but became higher when

priming was performed with DNAE7$_{GGG}$ (GB.1 group). A reduction in the antiviral IFN-γ response has already been shown for vaccinia and other mammalian poxviruses [135] that express genes mimicking the IFN-γ receptor, but this is unlikely for FP-based vectors that do not replicate in mammalian cells, and has never been described in FP-immunized animals [113]. To better define the specific induction of IFN-γ-secreting cells, other highly stimulatory antigens like OVA could be used, although other authors have already demonstrated that HPV E7 and OVA generate comparable results on T-cell response [55].

Finally, therapeutic immunization protocols using recombinants expressing the E6$_{F47R}$ or E7$_{GGG}$ genes were administered to mice previously challenged with TC-1* cells with the aim at evaluating the therapeutic efficacy of the different regimens.
After the therapeutic immunization with the recombinants expressing the E6$_{F47R}$ gene, tumor growth was delayed in mice of all the E6$_{F47R}$-immunized groups with 80% of still-healthy animals at 21 days p.c., when 100% of the mock-infected control animals showed tumor development. In particular, the numbers of healthy animals remained higher after the DNA/DNA protocol, which should therefore be considered the most effective regimen in mice. However, although genetic vaccination has emerged as an attractive strategy for immunotherapy, in studies performed in non-human and human primates, DNA vaccines suffered from low immunogenicity and efficacy. Previous studies have also demonstrated that higher protection can be obtained when animals are primed with recombinant DNA followed by recombinant FP boost than when using FP recombinants alone [79,120]. In the present project, both DNA/FP and FP/FP prime/boost immunization regimens were tested for the therapeutic immunization and a difference was noted between groups immunized with DNA/FP rather than FP/FP. This suggests that DNA prime followed by the FP boost

can be a more promising strategy to induce CTLs, kill tumor cells, and be effective in eliciting anti-tumor immune responses in humans.

After therapeutic immunization using the recombinants expressing the $E7_{GGG}$ gene on previously TC-1*-challenged mice, tumor development was delayed in DNA/FP immunized mice compared to those immunized twice with FP, with 50% of still-healthy animals 45 days p.c., whereas all of the mock-infected animals of the control group showed greater tumor development and a lower survival. Since Kaplan–Meier analysis did not indicate a significant statistical difference among the groups, it is not possible to exclude any random effect of the assay unless a higher number of animals is used. Although the use of a FP vector with unrelated antigens could be a more appropriate control, the experiment was repeated twice with similar results. This suggests that DNA prime followed by the FP boost can be a promising strategy, as FPE7 recombinants might better induce CTLs, which can destroy the tumor cells, and be more effective in eliciting anti-tumor immune responses in humans.

Many HPV vaccine strategies have focused on eliciting HPV E6/E7-specific T-cell responses, and several vaccination trials have been performed on patients with cervical cancer, genital warts and papillomas [136,137] using E6/E7 proteins, DNA or viral vectors carrying the E6/E7 oncogenes or their variants. Studies are also ongoing to improve antigen processing and presentation [138,139]. In this context, a partial tumor regression was obtained in the rabbit model by VSV-based vaccines encoding the E6 or UbE6 [140,141] and a potent antitumor effect was induced by administering mice with DNA vaccines encoding HPV16 E6, E7 and L2 proteins fused to calreticulin (CRT) [139]. Some of these vaccines, as CRT/E7(detox) and VGX-3100 [142], are also in clinical trial.

However, vaccines expressing the E6 gene alone have not previously been tested for therapeutic efficacy in prime/boost protocols.

Several therapeutic vaccination trials have also been performed on patients with cervical cancer, genital warts and papillomas [136,137] using the HPV16 E7 protein, DNA or viral vectors carrying the E7 oncogene or its variants. However, in spite of the immune responses, the expected efficacy was often low, suggesting that other effects have to be elicited by therapeutic vaccines, using adjuvants, innate immunity stimulation, or Treg depletion [143]. Vaccination with VV recombinants expressing the E7 oncogene elicited a strong response and has proven to be well tolerated in animal and human trials. These recombinants have promoted tumor regression in patients with advanced cervical cancer, which was sustained by the induction of CTLs specifically directed against infected cells [78,144].

Due to their natural host-range restriction to avian species [145,106], correct expression of transgenes in mammalian cells, and ability to elicit a complete immune response in vaccinated hosts [146], FP recombinants, which were used as vectors in this project, might represent alternative and safer immunogens to VV, which causes lytic infection, ulcerations, and scab formation. Moreover, the use of FP recombinants, combined or not with DNA in a prime-boost immunization regimens, has already shown higher protection when mice were primed with DNA followed by an FP boost [120].

In conclusion, during this project we demonstrated that: (i) $FPE6_{F47R}$ and $FPE7_{GGG}$ can correctly express the $E6_{F47R}$ and $E7_{GGG}$ proteins, respectively; (ii) anti-E6- and anti-E7-specific antibodies can be detected by ELISA, although at low levels, after mice immunization using recombinant E6 or E7 proteins or CaSki cell lysates as plated antigens; (iii) specific increase in IFN-γ-producing cell responses are induced in mice as

verified by the immune response protocols; (iv) after therapeutic immunization, the numbers of tumor-bearing mice are lower when the heterologous prime-boost regimens are used.

As future perspectives, results obtained during this project could represent a starting point to improve the development of an effective preventive/therapeutic vaccine to control HPV-related cervical cancers. In particular, the introduction of further modifications into the sequences of the E6 and E7 genes, such as their fusion with the Coat Protein derived from the Potato virus X, as well as the combined use of the two proteins into the same immunization regimen, could improve the immunogenicity, and thus the efficacy of future vaccines.

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