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**Breadth of T cell responses after immunization with adenovirus vectors encoding ancestral antigens or polyvalent papillomavirus antigens**

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Short title: Cross-reactive and polyvalent papillomavirus vaccines

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## Abstract

Oncogenic human papillomaviruses (HPVs) are in most cases eliminated by intervention of T cells. As many other pathogens, these oncogenic HPVs belong to an ancient and diverse virus family. Therefore, we found it relevant to investigate the potential and limitations of inducing a broad response - either by inducing cross-reactive T cells or by administering a polyvalent vaccine. To test these strategies, we designed 3 ancestral and 2 circulating sequences based on the two domains of the E1 and E2 proteins of papillomaviruses (PVs) that exhibit the highest degree of conservation in comparison to the other PV proteins. The PV sequences were fused to a T cell adjuvant, the murine invariant chain and encoded in a recombinant adenoviral vector which was administered to naïve outbred mice. By measuring T cell responses induced by these different vaccines and towards peptide pools representing 3 circulating strains and a putative ancestor of oncogenic HPVs, we showed that the ancestral vaccine antigen has to be approximately 90% identical to the circulating PVs before a marked drop of ~90% mean CD8+ T cell responses ensues. Interestingly, the combination of two or three type-specific PV vaccines did not induce a significant decrease of the CD8+ T cell response to the individual targeted PV types. Polyvalent HPV vaccine based on the E1 and E2 proteins seem to be capable of triggering responses towards more than one type of PV while the cross-reactivity of ancestral vaccine seems insufficient in consideration of the sequence diversity between HPV types.

## Introduction

In most cases pathogens belong to a very diverse and large family as it is the case for HIV, HCV, HPV and *Plasmodium falciparum*. Therefore developing vaccine strategies that can trigger more than one strain of pathogen has been a major focus in the vaccinology field. For that purpose, researches have been mostly focuses on either developing cross-reactive vaccines using consensus/ancestral sequences or developing polyvalent vaccines. Induction of cross-reactivity is appealing as one vaccine can target many types of viruses within the family, but the difficulty to induce such a response increases with the degree of diversity. A polyvalent vaccine might then be more feasible, but variability of responses to the individual types in the mixture may ultimately limit the number of targeted types that can be included in such vaccines.

Oncogenic human papillomaviruses (HPVs) are among the viruses that belong to a highly diverse family [1]. Until now three prophylactic HPV vaccines (Gardasil, Gardasil-9, Cervarix) have been licensed. They are polyvalent vaccines and Gardasil-9 that has been recently introduced can induce responses towards 9 different HPV types [2]. Despite their nearly complete efficacy against vaccine targeted papillomavirus (PV) types in naïve women [3], developing therapeutic vaccines is yet an important focus in the scientific literature because of the low uptake of the preventive vaccines [4] and their poor therapeutic efficacy [3]. Therefore, exploring the possibility to develop either a cross-reactive T cell based vaccine or a polyvalent vaccine has additional relevance for combating HPV infections.

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For attempts to induce cross-reactive responses and stimulate normal HPV elimination mechanisms, we focused on the E1 and E2 proteins, which are the most conserved among the HPV early proteins. Importantly, the selection of the E1 and E2 proteins also has consequences for the clinical targeting potential of a vaccine. The E1 and E2 early proteins of HPVs are involved in the replication of the virus genome and the regulation of E6 and E7 oncogenic proteins [5]. As these two proteins are highly expressed during chronic infection and low-grade cervical intraepithelial neoplasia (CIN) [6, 7] we are limiting ourselves to targeting the early stage of the infection before the cancer is established. This is in contrast to the majority of therapeutic strategies that targets the E6 and E7 oncogenes and therefore target the more advanced lesions and cancers [5, 8]. Notably, it is at the early stages when most spontaneous regression occurs [9] and we therefore anticipate that enhancing this regression rate is more feasible than achieving efficacy against malignancy. Regression is correlated with T cell infiltrations in warts [10] and with E2 specific T cell responses in previously exposed, now uninfected children [11] and therefore vaccination at this time could potentially boost the natural immunity to HPV for infected women.

We used Bayesian methods to reconstruct phylogenic trees and ancestral sequences having different degrees of similarity to the circulating sequences. The ancestral sequences selected for functional studies included one corresponding to the root of a phylogenetic tree of all oncogenic human and macaques PVs (CDSE1E2 antigen) as well as two other sequences that were ancestral to the clades containing HPV16/31/35 and HPV18/45 respectively. We also designed two vaccines encoding circulating human and macaques PVs sequences (HPV16E1E2, MfPV3E1E2). The E1 and E2 sequences of *Macaca fascicularis* type 3 (MfPV3)

were also included as it had been shown to be closely related to HPV16 and associated with both persistence and intraepithelial neoplasia [12, 13], thus MfPV3 would potentially offer a test model for vaccines against persisting PV infection.

In this study, each of our designed antigens (ancestral and circulating) was fused to a T cell adjuvant, the murine invariant chain (mli), encoded in the replication deficient human adenovirus type 5 (Ad5) and tested in naïve outbred mice. Fusion of the MHC class II associated invariant chain (li) to the vaccine antigen leads to an increase of the antigen presentation on the surface of transduced dendritic cells (DCs) which has been correlated with an increase of the CD4+ and CD8 + T cell proliferation and a significant rise of the magnitude of the CD8+ T cell responses independent of the CD4 help [14]. Since the design of this novel technology in 2005-2008, it has been used with different antigens and tested in different strains of mice and primates [14-16] and has been highlighted by others to be among the best described T cell adjuvants for adenovirus vectored vaccines [17]. We have never observed reduced responses to li linked antigen as compared to unlinked antigen [14, 15, 17, 18] and in a recent study, Capone et al. (2014) showed that chimpanzee adenovirus vector encoding the non-structural (NS) protein of the genotype 1b of HCV was able to induce a relatively similar T cell response to the NS protein of the HCV genotype 3a. Importantly, the breadth of the detectable vaccine induced response was enhanced by fusing the protein to the murine invariant chain (mli) [15]. Therefore, the mli is a relevant tool to include in our vaccine constructs and may increase the detection of low magnitude responses that would go unnoticed without the use of the invariant chain sequence.

By using the CDSE1E2, HPV18\_45E1E2, HPV16\_31\_35E1E2, MfPV3E1E2, HPV16E1E2 sequences and peptide pools targeting E1 and E2 from CDS, HPV16, HPV18, MfPV3 we showed that ancestral sequences based on the E1 and E2 proteins of papillomaviruses had to share close to 90% homology to the circulating PVs in order to induce a consistent T cell response at a level similar to the vaccine antigen. Vaccines with less similarity to the applied peptide pools rapidly dropped in response magnitude and consistency in outbred mice. We also showed that the T cell responses specific to each vaccine antigen that was induced after vaccination with up to three vaccines at once were similar to the T cell responses after immunization with one vaccine. A vaccine cocktail or a polygenic vaccine encoding up to three antigens could therefore theoretically achieve targeting of a large fraction of the circulating oncogenic strains.

## **Materials and Methods**

### **Mice**

Female CD1 mice were obtained from Scanbur (Denmark) and were acclimated for at least one week prior to vaccination. For all experiments mice were between seven and nine weeks of age at the start. The experimental procedure was approved by the national ethics committee on experimental animal welfare (dyreforsøgstilsynet) and performed according to the Danish guidelines.

### **Generation of the different PVs ancestral sequences**

First, multiple alignments for the E1 and E2 protein of the most frequent HPVs were performed using the software MAFFT [19]. For each alignment, the best fitting amino acid substitution models was then determined using the ProtTest3 software [20]. For

both protein data sets this was found to be JTT + I + G. Phylogeny and ancestral sequences were then inferred using the software MrBayes v3.1.2 [21]. For each data set the program was run for at least 3,000,000 generations, with two parallel runs of 5 chains each. Convergence of the Markov chain Monte Carlo run was checked using the software Tracer (Tracer v1.6, available <http://beast.bio.ed.ac.uk/Tracer>) and also by using the convergence diagnostics output by MrBayes. Finally, we used software written by the authors to automatically infer and remove subsequences corresponding to inserts that most likely were not present in a given ancestral sequence. Once the ancestral sequences were generated, the relatively conserved N- and C- terminal domains of E1 and E2 were selected and separated by GS linkers. The sequences are listed in the supplementary figure 1.

The CDSE1E2 has also been applied in a primate immunogenicity and efficacy study against *Macaca fascicularis* papillomaviruses (Ragonnaud et al., manuscript accepted, December 2016)

### **Adenoviral vector production and immunizations**

The above ancestral antigens (CDSE1E1, HPV16\_31\_35E1E2, HPV18\_45E1E2) and the PV circulating antigens (HPV16E1E2 and MfPV3E1E2) were subcloned directly after the full length of the murine invariant chain (mli) within the same ORF. The mli was in a transgene expression cassette of a shuttle plasmid and flanked by a CMV promoter (cytomegalus promoter) with tetracycline operator (TetO) sites [22] and a SV40 polyA (simian virus 40 poly A signal). The cassette was then inserted into the E1 region of an E1/E3-deleted recombinant human adenovirus type 5 (Ad5) genomic plasmid by homologous recombination in BJ5183. The newly generated plasmid containing the Ad5 virus genome with the relevant sequence in the E1 locus



was linearized and transfected in T-Rex-293 cell line (Thermo Scientific). Each virus was propagated in this cell line and purified by CsCl gradient ultracentrifugation as previously described [23]. The genome of the purified viruses was isolated and genomic DNA sequenced to verify the newly inserted sequence into the E1 deleted locus and digest by restriction enzymes digestion for further quality control. The number of virus particles (VPs) was identified by measuring the OD value of the purified virus on a NanoDrop-2000 (Thermo Scientific). The infectious virus titer (IFU) for each vaccine was determined at the same time with the Adeno-X Rapid Titer system (Clontech, Mountain View, CA).

All mice were immunized subcutaneously (s.c.), behind the foot pad in the right leg with  $2 \times 10^7$  IFU of the corresponding vaccines in 30  $\mu$ l of PBS as in a number of previous studies using li adjuvant vaccines[14, 18, 24-27].

### **Peptides**

Peptide pools used in this study contained 96 peptides for the CDSE1 protein, 42 peptides for the CDSE2 protein, 94 peptides for the HPV16E1 protein, 41 peptides for the HPV16E2 protein, 97 peptides for the HPV18E1 protein, 40 peptides for the HPV18E2 protein, 96 peptides for the MfPVE1 protein, 39 peptides for the MfPV3E2 protein. In order to span the entire sequence of each protein, peptides were 16 a.a. long and overlap by 11 a.a. These different peptides were purified and pooled by JPT (Germany) and shipped to Denmark in a lyophilized form.

## **Flow cytometry**

Spleens were collected from mice 14 days after immunizations and each of them was mashed through a net to obtain a single cell suspension that was then stimulated at 37°C and 5% CO<sub>2</sub> for 5 hours with 1 µg/ml of relevant peptide pools and 3 µM of Monensin. After incubation, cells were stained according to the standard protocols [28, 29]. Briefly, cells were first stained using the following surface anti-mouse antibodies (Biolegend): CD8\_PerCP.Cy5.5, CD4\_Pe, CD44\_FITC; then fixed using a 1% paraformaldehyde (PFA) solution and finally permeabilized to allow the entry of the anti-mouse IFN-γ<sub>APC</sub> antibody (Biolegend). The data were collected on the LSRII and FORTRESSA instruments (BD Biosciences).

## **Determination of the percentage of identity between the vaccine antigens and the proteins used for splenocyte stimulation**

The percentage of identity between the vaccines sequences and the proteins sequences (used to produce the different peptide pools for the flow cytometry assay) was calculated by multi-alignment using the algorithm “blastp” on the NCBI website.

## **Statistical analysis**

Graphs and statistical analysis were performed using Prism software (version 5). Quantitative results were compared using two-tailed Mann\_Whitney (non-parametric) test. p Values (p) > 0.05 were nonsignificant (n.s.) values, p ≤ 0,05 were denoted “\*”, p ≤ 0,01 were denoted “\*\*” and p ≤ 0,001 were denoted “\*\*\*”.

## Results

### **Amino acid sequence homologies between the vaccine antigens and PVs proteins**

After phylogenetic reconstruction based on the E1 and E2 proteins of oncogenic HPVs, we selected three ancestral sequences (CDSE1E2, HPV16\_31\_35E1E2 and HPV18\_45E1E2) that correspond to different internal nodes in the phylogenetic tree, as well as two existing PV sequences (MfPV3E1E2 and HPV16E1E2). These different sequences (listed in the supplementary figure 1) were fused to the T cell adjuvant, the murine invariant chain [14] and encoded in a replication deficient adenovirus type 5 (Ad5) as illustrated in figure 1A (Fig. 1A).

Using standard multiple-alignment tools we identified the percentage of amino acid homologies between the vaccine antigen sequences and the peptide pools covering the E1 and E2 proteins from ancestral and circulating PVs (Fig. 1B). Through these different combinations we cover a distance from 50 % to 100 % identity providing us with a novel platform to study cross-reactivity based on the E1 and E2 proteins.

### **A high percentage of identity between ancestral and circulating PVs amino acid sequences is necessary to induce robust cross-reactive CD8+ T cell responses**

We next investigated the ability of our different vaccine constructs to induce cross-reactive T cell responses. For that purpose, outbred mice were vaccinated with one of the 5 vaccines and 14 days later, we analyzed the CD8+ T cell responses towards peptide pools covering the E1 and E2 proteins of CDSE1E2, HVP18, HPV16 and MfPV3. Here, each vaccinated group was able to induce a strong CD8+ T cell response towards the E1 vaccine-encoded antigen in an average of  $6 \times 10^5$  IFN- $\gamma$

producing CD8<sup>+</sup> T cells (Fig. 2A). Only the vaccine encoding the ancestral sequence corresponding to the internal node closest to HPV18 and HPV45 (Ad5\_mli\_HP18\_45E1E2) and sharing about 92% of identity to the E1 protein of HPV18 was able to raise a similar HPV 18 specific CD8<sup>+</sup> T cell response in all vaccinated mice as it was against homologous vaccine antigens (Fig. 2A and B). When the percentage of homology was lower than 92 %, some, but not all, vaccinated mice were able to raise cross-reactive CD8<sup>+</sup> T cell responses and for those responding mice the responses were significantly lower than against vaccine antigens with an average drop of about 10 fold (Fig. 2A and B). The CD8<sup>+</sup> T cell responses raised against the E2 protein were not as consistent as against the E1 protein. Certainly, not all vaccinated mice were able to induce CD8<sup>+</sup> T cell responses against the E2 protein encoded in the vaccines, and this antigen was also found to provide poor or non-existent cross-reactivity (Fig. 2C and D). Occasionally potent responders were detected including quite prominently for the 76% homology group (Fig. 2 D). To get an idea of the overall responses, we added the percentage of the IFN- $\gamma$  producing CD8<sup>+</sup> T cells after E2 stimulation to the percentage after E1 stimulation. This did not result in a higher number of responders which can be explained as the few E2 responders had also responded to the E1 protein (Fig. 2E and F).

### **Combination of up to three PV vaccines does not negatively affect the induced CD8<sup>+</sup> T cell responses**

The magnitude of the PV-specific T cell responses induced after vaccination in order to eliminate PV infections is still unknown. Therefore, we found it interesting to investigate whether the combination of two or three vaccines could maintain the magnitude and/or consistency of the T cell responses specific to the vaccine-

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encoded antigens. For that purpose we immunized 3 groups of outbred mice, one group with the Ad5\_mli\_MfPV3E1E2 vaccine, a second group with the Ad5\_mli\_MfPV3E1E2 and Ad5\_mli\_HP16E1E2 vaccines and a last group with the Ad5\_mli\_MfPV3E1E2, Ad5\_mli\_HP16E1E2 and Ad5\_mli\_CDSE1E2 vaccines. As in the previous experiment, we analyzed the CD8<sup>+</sup> T cell responses 14 days post vaccination from mouse spleen. As seen in figure 3A, all vaccinated groups were able to raise a similar CD8<sup>+</sup> T cell response to the E1 protein of MfPV3, an average of  $2 \times 10^5$  IFN- $\gamma$  producing CD8<sup>+</sup> T cells were induced in each vaccinated group. Therefore combination of up to three vaccines did not have any effect on the MfPV3E2 specific CD8<sup>+</sup> T cell responses. The last group of mice that received 3 vaccines including the Ad5\_mli\_CDSE1E2 vaccine was also able to induce a potent CD8<sup>+</sup> T cell response to the E1 protein of CDSE1E2 antigen (Fig. 3A) that was similar to the group of mice that received only this vaccine in the previous experiment (Fig. 2A). A slight, but not statistically significant, decrease of the E1 HPV16 specific CD8<sup>+</sup> T cell response was observed in the group of mice that received 3 vaccines in comparison to the combination of the two vaccines (Fig 3A), but all animals responded against all antigens used for immunization. As seen in the previous experiment, the E2 specific CD8<sup>+</sup> T cell responses induced by any of the vaccines was low in every group (Fig. 3B).

### **Similar conclusions concerning the CD4<sup>+</sup> T cell responses**

Adenoviral vaccines, even without using the li adjuvant, are first and foremost potent stimulators of CD8<sup>+</sup> T cell responses [30, 31]. Nevertheless, the CD4<sup>+</sup> T cells have been shown to have an important role in the regression of HPV infections [10, 23, 32, 33]. Therefore, we also analyzed the CD4<sup>+</sup> T cell response after vaccination at the same time as the CD8<sup>+</sup> T cell response was measured. The vaccine-induced

CD4+ T cell responses were found to be much lower than the CD8+ T cell responses and we therefore decided to focus on the total E1 and E2 specific CD4+ T cell responses. In this analysis the results for each experiment were similar to the CD8+ T cell responses and again, a minimum of 90% of homology between the PV ancestral sequences and the circulating PVs was necessary to induce a CD4+ T cell response similar in magnitude to the vaccine antigen-specific CD4+ T cell response (Fig. 4A and B). Also, the combination of up to three vaccines was still as potent as a single vaccine against the same specificity (Fig. 4C).

## Discussion

The aim of the study was to investigate the possibility to design a therapeutic adenoviral vaccine that can elicit T cell responses specific to more than one type of circulating oncogenic papillomavirus. For that purpose, two approaches were tested. The first one was to analyze the induced cross-reactive T cell responses after vaccination with adenoviral vectors encoding E1 and E2 ancestral sequences located at different distances to circulating PVs and to identify the similarity requirement for induction of consistent responses. The second approach was to investigate the possibility of combining several vaccines and observe if we still preserve the T cell responses to each targeted PVs.

When we investigated the ability of these different ancestral vaccines at inducing cross-reactive T cell responses, we discovered that at least 90% of amino acid identity between the vaccine antigen and the circulating PV strains was necessary in order to match vaccine antigen-specific T cell responses. A percentage of homology lower than 90% induced a significant reduction of the cross-reactive T cell-responses towards targeted PVs and more variation within the responding mice with a high

frequency of non-responders. For the prospects of using ancestral sequences to generate very broadly effective vaccines against PVs, this lack of consistency was a sobering finding, but it may be sufficient for cross-reactive vaccines against less diverse pathogens such as HIV-1 with 90 to 85 % of homology between clades in the major structural proteins pol and gag respectively [34]. The rather strict homology-requirement we observed as necessary to maintain robust T cell responses is superficially different from the Capone et al. study where only 70% identity (as determined by multiple alignment of the amino acid sequences) between the two non-structural (NS) proteins was shared and where cross-reactivity was found in all of the vaccinated outbred mice [15]. As we used the human adenoviral vector type 5 that has shown similar potency to the chimpanzee adenoviral vectors [30] and the mli which was also used in the Capone et al. study [15], our platform should have been performing similarly. A potential factor to explain the discrepancies is the size of our E1E2 antigen that is much smaller than the NS protein used in Capone et al. [15]. A shorter antigen will arguably tend to have fewer MHC binding peptides and therefore less epitopes of which a fraction have the capacity to inducing cross-reactive epitopes. Looking at the individual responding animals in Capone et al. [15] it is also evident that the animals responded with heterogeneity and the animals had responses towards 4 to 6 of 6 included homologous peptide pools. Our antigen (length ~700 a.a.) is considerably shorter than the NS antigen (length ~2000 a.a.), and in this light it may not be surprising that we saw less homogeneity in the responses and only occasionally responses towards the E2 protein (~200 a.a.) in comparison to the E1 protein (~500 a.a.). Also, if our vaccines induce responses to fewer epitopes it is less of a surprise that it sometimes fail completely against strains with more than 10% of dissimilarity. Notably, the mean of the responses we

observed were approximately 10-15 folds lower which is not too different from the responses in Capone et al. [15]. Additionally, it is possible that we, by selecting the most conserved regions of the E1 and E2 proteins, may also have selected protein domains that are less immunogenic than the highly diverse regions. Such adaptation has been recently reported to have happened during the course of HIV evolution [35]. Such adaptation might also help explain why the E2 protein was poorly immunogenic in most measurements in the study. As a curiosity in our dataset, we did observe the group of 76% of E2 homology responding more potently than the group of 83-86 % of homology, but the group of 76 % of is also the smallest data set and it might be a chance event.

Importantly, while the ability to eliminate PV infections has been shown to be genetically linked to genes within the immune system [23] and correlated with T cell infiltration [10], the PV specific T cell responses appear difficult to detect in the blood [36]. Therefore, a low cross-reactive T cell responses induced after vaccination with an adenovirus vector encoded the PV ancestral sequence might be sufficient to eliminate oncogenic PV infections, if it could only be consistently induced.

In this study, as an alternative to the ancestral antigen design we also showed that it was possible to combine up to three PV vaccines while preserving the T cell responses to each of the included PV antigens. A vaccine cocktail or a polygenic vaccine encoding up to three antigens could theoretically achieve targeting of a large fraction of the circulating oncogenic strains. These data are different from the results reported in the Larke et al. study (2007) which showed that combining single-clade HIV-1 vaccines reduced the breadth of induced T cell responses, although simultaneous injection into anatomically separated sites could increase these T cell responses [37]. We did not apply different injection sites to avoid immunodominance



between the antigens, as this defies the purpose of combining the vaccines to make a useful polyvalent vaccine formulation, but we nevertheless achieved strong responses to each included antigen. This could be a benefit of the li adjuvant that has demonstrated improved capacity to compete with vector derived immune responses for induction of broader responses [38].

From our findings it is clear that a single short ancestral sequence cannot induce the coveted consistent E1 and E2 specific responses, and that relatively close sequence similarity is needed, but we have obtained clues to how this could be achieved. Simply mixing vaccine vectors seems to be a viable strategy and would presumably induce broad if not exactly global coverage. When analyzing the differences and similarities to the study by Capone et al. [15] inclusion of larger fragments of the papillomavirus genomes and potentially encoding multiple antigens could be seen as logical steps forward.

Accordingly, the information provided in this study may bring us closer to obtain a therapeutic vaccine that can trigger more than one type of papillomavirus. Furthermore, the basic conclusions obtained may find relevance for vaccination attempts against other variable T cell antigens.

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## Figure Legends

**Fig 1. Adenovirus vaccines and amino acid distances between the vaccine antigens and papillomavirus proteins.** (A) A schematic overview of replicative deficient adenovirus vaccines expressing either ancestral (CDS, HPV16\_31\_35, HPV18\_45) or circulating (MfPV3, HPV16) of the N (Nterm) and C (Cterm) terminal domain of the E1 and E2 proteins of papillomaviruses (PVs). Each of these sequences is fused to the murine invariant chain (mli). The number of amino acid (a.a.) is written below each protein. (B) Tables illustrating the percentage (%) of a.a. homology between the different vaccine antigens and the E1 (top table) and E2 (bottom table) proteins of the ancestral and circulating PVs. The % of homology was identified by multi-alignment (blastp) on the NCBI website.

**Fig 2. Cross-reactive CD8+ T cell responses induced by adenoviral vectors.** Group of 5 CD1 mice were vaccinated with one the adenovirus vaccines at  $2 \times 10^7$  infectious unit (IFU). All mice were euthanized 14 days later and total number of splenic CD8+ T cells responding to with IFN- $\gamma$  production to peptide pools of the ancestral (CDS) and circulating (HPV16, HPV18, MfPV3) E1 and E2 proteins of PVs (A, C and E). These T cell responses were grouped depending on the distances

(% of a.a. homology) between the vaccines' antigen and the peptide pools of the E1 and E2 proteins (B, D and E). These distances were characterized in the previous figure. The E1E2 T cell responses (E and F) correspond to the addition of the % of IFN- $\gamma$  CD8<sup>+</sup> T cells responding to the E1 pools and to the % of IFN- $\gamma$  CD8<sup>+</sup> T cells responding to the E2 pools. Splenocytes were gated on CD8<sup>+</sup>, CD44<sup>+</sup>, and IFN- $\gamma$ <sup>+</sup> T cells. Each dot and bar represents a mouse and the mean respectively. This experiment was performed twice and the data were pooled. "n.s." denotes for nonsignificant.

**Fig 3. CD8<sup>+</sup> T cell responses after vaccination with up to three adenoviral vectors.** Group of 6-10 CD1 mice were vaccinated either with one vaccine (Ad5\_mli\_MfPV3E1E2) or two vaccines (Ad5\_mli\_MfPV3E1E2, Ad5\_mli\_HP16E1E2) or three vaccines (Ad5\_mli\_MfPV3E1E2, Ad5\_mli\_HP16E1E2, Ad5\_mli\_CDSE1E2) at  $2 \times 10^7$  IFU for each vaccine. All mice were euthanized 14 days later and analysis of splenic CD8<sup>+</sup> T cell responses was performed by IFN- $\gamma$  intracellular cytokine staining after in vitro stimulation with peptide pools of the ancestral and circulating E1 (A) and E2 (B) PVs proteins. Splenocytes were gated on CD8<sup>+</sup>, CD44<sup>+</sup>, and IFN- $\gamma$ <sup>+</sup> T cells. Each dot and bar represents a mouse and the mean respectively. "n.s." denotes for nonsignificant. The experiment was performed once.

**Fig 4. CD4<sup>+</sup> T cell responses after vaccination with adenoviral vectors.** E1E2 specific CD4<sup>+</sup> T cell responses were analyzed at the same time as the CD8<sup>+</sup> T cell responses from the 2 previous experiments. (A) and (B) refer to the experiment illustrated in figure 2 and (C) refer to the experiment illustrated in figure 3. As it was mentioned on the figure 2 legends, the E1E2 T cell responses correspond to the

addition of the % of IFN- $\gamma$  CD8+ T cells responding to the E1 pools and the % of IFN- $\gamma$  CD8+ T responding to the E2 pools. “n.s.” denotes for nonsignificant.

**Supplementary figure 1. Amino acid sequences of the different antigenic constructs: CDSE1E2, HPV16\_31\_35E1E2, HPV18\_45E1E2, HPV16E1E2 and MfPV3E1E2.** The sequences were constructed with the following orientation of the papillomavirus genes and GS linkers: E1Nterm-GS-E1Cterm-GS-E2Nterm-GS-E2Cterm. The GS linkers are highlighted in bold and underlined.

Figure 1

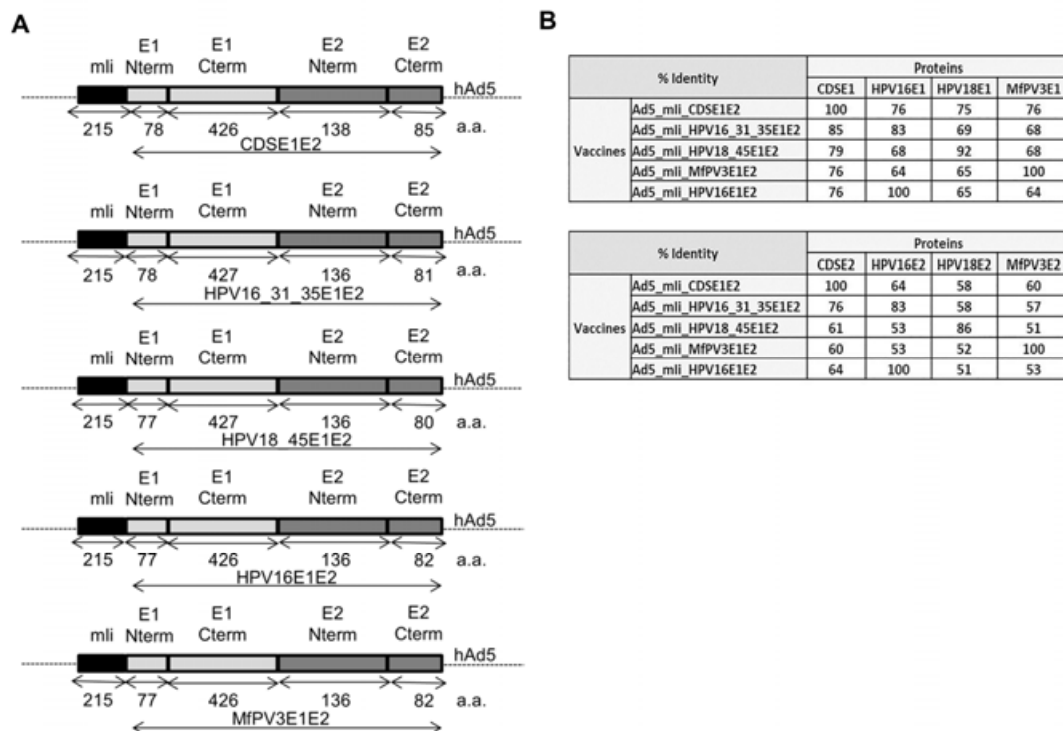


Figure 2

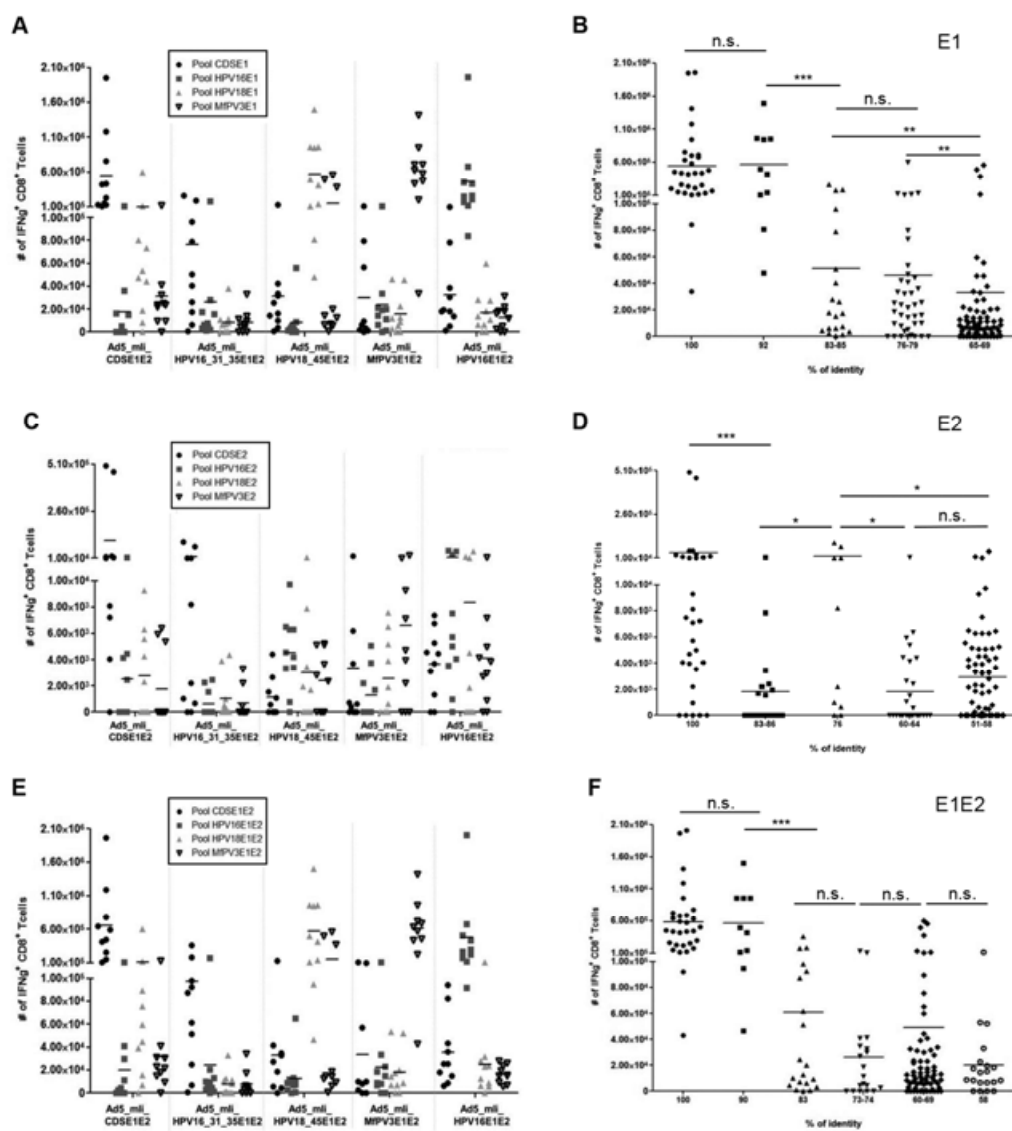


Figure 3

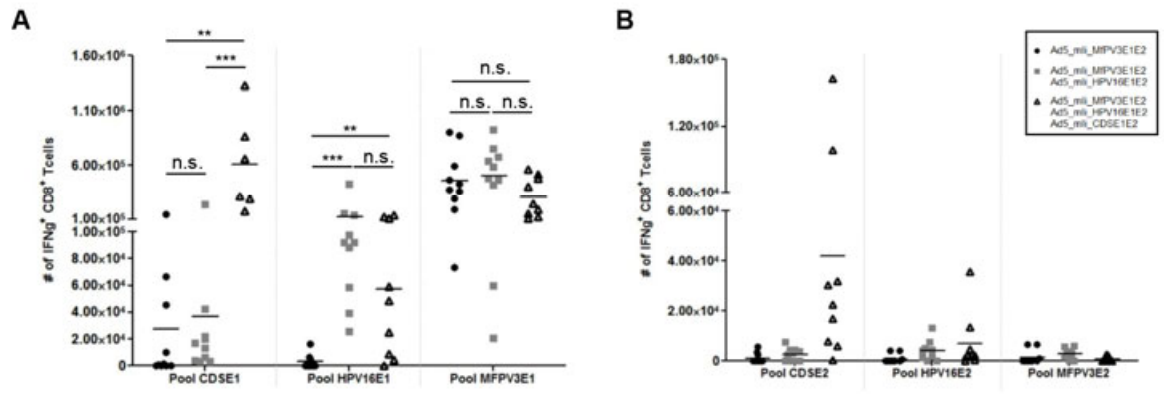




Figure 4

