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Adenovirus vector-based prime-boost vaccination via heterologous routes induces cervicovaginal CD8+ T cell responses against HPV16 oncoproteins

Nicolas Çuburu[1], **Selina Khan**[2], **Cynthia D Thompson**[1], **Rina Kim**[1], **Jort Vellinga**[2], **Roland Zahn**[2], **Douglas R Lowy**[1], **Gert Scheper**[2], and **John T Schiller**[1]

[1]Laboratory of Cellular Oncology, National Cancer Institute, National Institutes of Health, Bethesda, Maryland, MD, USA [2]Janssen Vaccines & Prevention, Leiden, Netherland

Abstract

Recent advances in immunotherapy against cancer underscore the importance of T lymphocytes and tumor microenvironment, but few vaccines targeting cancer have been approved likely due in part to the dearth of common tumor antigens, insufficient immunogenicity and the evolution of immune evasion mechanisms during the progression to malignancy. Human papillomaviruses (HPV) are the primary etiologic agents of cervical cancer and progression from persistent HPV-infection to cervical intraepithelial lesions and eventually cancer requires persistent expression of the oncoproteins E6 and E7. This offers the opportunity to specifically target these virus-specific antigens for vaccine-induced clearance of infected cells before cancers develop. Here we have evaluated the immunogenicity of Adenovirus types 26 and 35 derived vectors expressing a fusion of HPV16 E6 and E7 oncoproteins after intramuscular and/or intravaginal immunization in mice. The adenovirus vectors were shown to transduce an intact cervicovaginal epithelium. Intramuscular prime followed by intravaginal boost maximized the induction and trafficking of HPV-specific $CD8^+$ T cells producing IFN-γ and TNF-α to the cervicovaginal tract. Importantly, the cervicovaginal $CD8^+$ T cells expressed CD69 and CD103, hallmarks of intraepithelial tissue resident memory $CD8^+$ T cells. This prime/boost strategy targeting heterologous locations also induced circulating HPV-specific $CD8^+$ T cell responses. Our study prompts further evaluation of intravaginal immunization with adenoviral vectors expressing modified E6 and E7 antigens for therapeutic vaccination against persistent HPV infection and cervical intraepithelial neoplasia.

Introduction

Cervical cancer is responsible for the death of 250,000 women each year and remains the third most common cancer in women worldwide[1]. Persistent Human papillomavirus (HPV) infection of the cervicovaginal mucosa with high-risk types is the central cause of cervical intraepithelial neoplasia of increasing severity (CIN1 to CIN3) and cervical cancer[2]. In addition, HPV infection causes a significant number of vulvar, penile, anal and oropharyngeal cancers. Transformation of cervicovaginal keratinocytes requires maintained expression of the oncoproteins E6 and E7[3]. Most HPV infections are cleared naturally and a significant fraction of CIN2/3 lesions regress overtime[4, 5]. It has been proposed that infiltration of high grade lesions by mucosal T cells is a good predictor of lesion regression

whereas exclusion of T cells from the lesions is associated with persistence of intraepithelial disease[6]. In this line it was shown that the programmed death-ligand 1 (PD-L1), an inhibitor of T cell receptor signaling, was up regulated even in low grade lesions (CIN1)[7]. These observations suggest that a therapeutic vaccine against persistent HPV infections and intraepithelial lesions should target viral antigens and induce cell-mediated immunity at the lesion site.

Three prophylactic VLP vaccines are used against HPV infections and prevent the occurrence of HPV-induced neoplasia[8]. They confer protection through the induction of neutralizing antibodies against the HPV capsid major protein L1. Once HPV persistent infection is established there is no evidence that these vaccines work in a therapeutic manner. In this context, incomplete vaccine coverage or acquisition of HPV infection before prophylactic vaccination will leave a significant number of women diagnosed with high-risk type HPV persistent infection and/or CIN. Promising results from therapeutic HPV vaccine clinical trials in suggest that a non-surgical alternative to the current treatments of high-grade lesions is achievable[9, 10]. In addition, a therapeutic vaccine could also provide a new intervention for women with persistent high-risk HPV infections and low-grade lesions and have a major public health impact in low resource settings when surgical procedures are unavailable.

Replication-defective viral vectors are attractive vehicles for genetic vaccination. They are intrinsically immunogenic, versatile platforms to encode various antigens, and may have a better safety profile than live attenuated viral vectors. Technologies based on adenoviruses (Ad) are the most advanced for genetic vaccination. Attractive features of Ad vectors are their ability to induce strong systemic T cell responses along with high serum antibodies after intramuscular (IM) immunization. Ad5 is the most common human serotype and despite highly prevalent type-specific neutralizing antibodies[11], it has been historically the most utilized serotype as a vaccine vector. In spite of unsatisfactory results for HIV vaccination with Ad vector serotype 5 (Ad5) based vaccines[12], technologies based on multiple Ad serotypes have shown promises for malaria, tuberculosis and Ebola virus vaccination[13]. Animal or less prevalent human Ad serotypes such as 26 (Ad26) and 35 (Ad35) have been developed to overcome type-specific anti-vector immunity induced by infection and/or vaccination[14]. Ad vectors have been used in numerous vaccine clinical trials alone against Ebola virus and tuberculosis or in prime/boost immunization regimens with other vaccine platforms, such as DNA or Modified Ankara virus (MVA) against HIV, malaria and respiratory syncytial virus[13]. Although Ad viruses are transmitted through mucosal sites (ocular, oral or respiratory) depending on the serotypes, the potential of Ad vectors for mucosal antigen delivery as vaccines remains to be explored in clinical settings.

Trafficking of $CD8^+$ T cells to the cervical lesions is likely to be a critical parameter for success of a therapeutic HPV vaccine. However, with few exceptions, most clinical trials of HPV-therapeutic vaccines have evaluated vaccine immunogenicity in the blood compartment[15]. Tissue resident memory $CD8^+$ T cells (Trm) cells have emerged recently as key players in antiviral immunity at mucosal surfaces and constitute a separate population from other circulating $CD8^+$ T cell memory populations[16, 17]. In previous studies, we have shown that intravaginal (Ivag) prime/boost immunization with non-replicating HPV viral

vectors was far superior to remote IM or intranasal immunizations with the vectors to induce high numbers of tissue resident memory $CD8^+$ T cells in the cervicovaginal mucosa[18, 19]. In addition, Ivag immunization with HPV vectors was superior to confer protection in genital challenge with vaccinia virus or Herpes simplex virus. This increased propensity of Ivag immunization with HPV vectors to induce CD8 Trm is even more remarkable given the fact that IM immunization is far more potent than Ivag immunization to induce systemic and circulating $CD8^+$ T cell responses.

Here we evaluated in mice a prototype therapeutic vaccine against HPV infection and neoplasia based on Ad26 and Ad35 expressing a fusion protein of HPV16 E6 and E7 oncoproteins (E6E7fus). We evaluated Ivag gene delivery with Ad26 and Ad35 vectors expressing reporter genes in time course and tropism experiments. We show that Ad vectors can transduce efficiently the intact cervicovaginal epithelium even in the presence of the potent HPV inhibitor carrageenan[20]. We evaluated prime/boost immunization regimens via heterologous routes combining classical IM immunization and Ivag immunization. We show that systemic prime followed by local Ivag booster is the most promising combination to efficiently induce concomitantly circulating and cervicovaginal resident $CD8^+$ T cell responses. Furthermore, we show that Ad35 IM/Ad26 Ivag booster immunization induces E6 and E7 specific polyfunctional cytokine-secreting $CD8^+$ T cells, but no detectable E6 or E7-specific $CD4^+$ T cells, in the spleen and in the cervicovaginal mucosa.

Material and Methods

Viral vector design, production and characterization

E1/E3-deleted replication-deficient Ad26 and Ad35 expressing either GFP, Firefly luciferase, or HPV16 E6E7fus sequences under control of a CMV promoter were constructed, amplified, purified and characterized as described[21, 22]. Viral particle concentration was determined by optical density in the presence of SDS, and viral infectivity by TCID50 assay. Bioburden (MicroSafe, Millipore) levels and Endotoxin (MicroSafe, Millipore) levels met the preset release criteria for animal experiments.

Mice, immunization and sample preparation

C57Bl/6 and BALB/c female mice (8- to 10 weeks old) were purchased from Jackson laboratory and maintained in the animal care facilities at the National Cancer Institute (NCI) under specific-pathogen-free conditions. All procedures were reviewed and approved by the NCI Animal Care and Use Committee.

For IM immunization, 2.5×10^9 to 1×10^{10} Ad virus particles (vp) were diluted in 50ul PBS and injected in the quadriceps of anesthetized mice.

For Ivag instillation, mice were treated subcutaneously (SC) with 3mg Medroxyprogesterone acetate (Depoprovera) 5 days prior to instillation of viral vector. Five hours prior to instillation viral vector, mice were pretreated with 4% nonoxynol-9 (N-9) to expose the basement membrane or 2% carboxymethyl cellulose (CMC) to keep an intact epithelium. Ad viral vectors (2.5×10^9 to 1×10^{10} vp) were diluted in 2 % CMC or 1% ι-

carrageenan, a potent topical HPV microbicide; and a total volume of 20ul was instilled intravaginally using a displacement pipette (Gilson).

A detailed description of the dissection procedure and samples preparation is available in supplementary methods.

In vivo bioluminescence assay

In vivo bioluminescence was monitored using a Xenogen IVIS Imager from 8hrs to 144hrs after Ivag instillation of Ad26 or Ad35 vectors expressing the firefly luciferase gene. Briefly, 3 min after Ivag instillation of 20μl D-Luciferin (Sigma, 15mg/ml), anesthetized mice were imaged using a 60sec exposure time and medium binning. Data are expressed as photon per sec (p/s).

Confocal microscopy

The cervicovaginal mucosa tissues were excised and embedded in OCT (Tissue-Tek) and kept frozen at −80C. In some experiments frozen 6-μm longitudinal sections of the cervicovaginal mucosa mounted on glass slides were stained with alexa-647 anti-MHC-II antibody with an antifade reagent (Prolong Gold with Molecular Probes) containing 4,6-diamidino-2-phenylindole (DAPI). Confocal images were acquired at the Confocal Microscopy Core Facility, Center for Cancer Research, National Cancer Institute, NIH, with Zeiss ZEN software on a Zeiss LSM 780 Confocal system using a 40X oil immersion objective and 364-nm, 488-nm, and 543-nm lasers. Images were analyzed for GFP expression using Adobe Photoshop, and color channel levels were adjusted uniformly across images.

In vitro T cell stimulation

For in vitro stimulation, cells were incubated for 6hrs at 37C, 5% CO2 in RPMI medium supplemented with 10% FBS, Sodium Pyruvate, L-Glutamine and 2-mercaptoethanol. Media containing overlapping 15mer peptide libraries of HPV16 E6, HPV16 E7 or the immunodominant peptide $E7_{49\text{-}57}$ peptide in the presence of brefeldin A (BD Golgi plug) were used to stimulate E6- and E7-specific cytokine production by T cells. Medium alone or containing PMA/ionomycin were used as a negative and positive stimulation controls.

Tetramer staining, intracellular cytokine staining and flow cytometry analysis

For tetramer staining, cell suspensions were stained for 30 min at 4°C with an APC-conjugated H-$2D^b$/$E7_{49\text{-}57}$ tetramer (NIH Tetramer Core Facility, Atlanta, GA) and purified anti CD16/32 antibody to block FcR. The following fluorochrome-conjugated antibodies (Table S1) were added for another incubation 20 min at 4°C: Pacific Blue anti-CD3, APC-Cy7 anti-CD4, PE-Cy7 anti-CD44, PE-Cy7 anti-CD69 or PE-Cy7 anti-CXCR3, FITC anti-CD62L, PerCP-Cy5.5 anti-CD103, PE anti-CD127, and Pacific Orange anti-CD8.

For intracellular cytokine staining, cell suspensions were stained for 20 min with PE anti-CD8, PE.Cy7 anti-CD3, APC.Cy7 anti-CD4 and purified anti CD16/32 antibodies followed by viability assay (LIVE/DEAD Yellow, Invitrogen) and fixation in 4% paraformaldehyde.

Intracellular staining using FITC anti-IFN-γ, PERCP.Cy5.5 anti-IL2, APC anti-TNF-α was done in permeabilization buffer (PermWash, BD) for 30min at 4C.

Adenovirus neutralization assay

In vitro adenovirus neutralization assay was performed as previously described [23].

Statistical analysis

Non-parametric Mann Whitney U test was performed to determine the P values in experiments with two independent groups. For experiments with more than two groups, a non-parametric one-way analysis (Kruskal-Wallis H test) was performed. Data are shown as mean ± SEM and P value ≤ to 0.05 (*), 0.01 (**), 0.001 (***) and 0.0001 (****) was considered statistically significant (ns: not significant).

Results

Ad26 and Ad35 transiently transduce the cervicovaginal mucosa epithelium upon N-9 treatment

Few preclinical studies have examined delivery of Ad vectors (e.g. Ad5 serotype) through the vaginal route[24, 25]. We evaluated Ivag gene delivery with Ad vectors type 26 and 35 following a protocol developed for HPV pseudovirus genital infection[26]. This protocol requires disruption of the cervicovaginal epithelium with the spermicide N-9 to expose the cervicovaginal epithelium basement membrane and to facilitate access and binding of the viral particles. We reasoned that N-9 treatment prior to Ad vector Ivag instillation might also facilitate in vivo transduction and expression of genetically delivered antigens. Transduction of the cervicovaginal mucosa with Ad26 and Ad35 expressing luciferase was transient, peaked between 12hrs and 24hrs after vector instillation and returned to baseline by 48hrs (Fig. 1A, B). In spite delivering the same number of viral particles (2.5×10^9 vp), the peak luciferase signal was 20-times higher after Ad26 Ivag instillation compared to Ad35. Fluorescence imaging of the cervicovaginal mucosa at the peak of expression (16–24hrs) showed that the epithelium of the cervicovaginal mucosa was transduced with Ad26-GFP and to a lesser extent with Ad35-GFP (Fig. 1C). Immunofluorescence staining with MHC-II antibody rarely colocalized with GFP-positive cells suggesting predominant keratinocyte tropism of the Ad26 and Ad35 vectors (Fig. 1C).

Prime/boost immunization via heterologous routes with Ad26 and Ad35 concomitantly induces cervicovaginal and systemic $CD8^+$ T cell responses against HPV16 E7

IM immunization is the most utilized route of administration of Ad vectors, with Ad5 being the most utilized serotype. We showed previously that in spite of the remarkable systemic immunogenicity of Ad5 vectors given IM, they are relatively ineffective at inducing cervicovaginal $CD8^+$ T cell responses against a model viral antigen, in comparison to HPV vectors given Ivag[19]. Here, we evaluated Ad vectors prime/boost immunization via homologous or heterologous routes combining the classical IM route with the Ivag route. Specifically, we evaluated whether the vaccination route order influences the induction of cervicovaginal and systemic $CD8^+$ T cell responses against the HPV16 E7 immunodominant epitope in C57Bl/6 mice. Because the induction of type-specific neutralizing antibodies

against the vector capsid might impair booster immunization in mice, we alternated serotypes Ad26 and Ad35 for the prime and booster to allow comparison of route of administration without neutralizing antibodies interfering. HPV16 E7-specific $CD8^+$ T cell responses were analyzed by flow cytometry using H2-D^bE7_{49-67} tetramers and CD103 integrin antibody, as a marker for intraepithelial lymphocytes and tissue resident lymphocytes at muco-cutaneous sites (Fig. 2A). The analysis was performed on pooled groups based on the route of administration rather than the serotypes used. Homologous prime/boost immunization Ivag/Ivag induced low numbers of cervicovaginal $tetramer^+CD8^+$ T cells that were comparable to IM/IM immunization (Fig. 2B). However, only Ivag/Ivag immunization induced expression of the integrin CD103 (Fig. 2E, $P \leq 0.0001$). The frequency of E7-specific $CD8^+$ T cells in the spleen was higher after IM/IM immunization compared to Ivag/Ivag immunization ($P \leq 0.0001$), but they did not express CD103 (Fig. 2F and G).

Importantly, the order of route usage for heterologous prime/boost immunization was critical to induce cervicovaginal $CD8^+$ T cell responses against HPV16 E7. Compared to IM/IM prime boost, mice primed IM and boosted Ivag displayed higher numbers (Fig. 2B, $P \leq 0.05$) and percent of cervicovaginal E7-specific $CD8^+$ T and cells (Fig. 2C, $P \leq 0.001$ and Fig S1) but mice primed Ivag and boosted IM did not. The percentage of CD103 expression by E7-specific $CD8^+$ T cells was increased in mice primed IM and boosted Ivag compared to IM/IM ($P \leq 0.001$) but comparable if Ivag route was used for at least one immunization. The number of E7-specific $CD8^+$ T cells expressing CD103 was increased in mice immunized IM/Ivag compared to Ivag/IM ($P \leq 0.001$). The order of serotype usage did not play a significant role in the magnitude and tissue distribution of $CD8^+$ T cell responses and in the expression of CD103 by E7-specific $CD8^+$ T cells.

The order of the route of immunization but not the order of serotype usage affects differentially the induction of circulating $CD8^+$ T cell responses

We next interrogated the impact of heterologous versus homologous prime/boost immunization on priming and recall of circulating E7-specific $CD8^+$ T cell responses. Blood samples were collected 3 weeks after each immunization and, as seen in the spleen, the highest $CD8^+$ T cell responses were observed in groups immunized at least once IM with either Ad26 or Ad35. After a single IM immunization with Ad26 or Ad35, the mean frequency of $CD8^+$ T cells in blood was higher than after a single Ivag immunization but decreased by two-fold after a month (Fig. 3A–B). In IM primed animals, a single IM booster with Ad26 or Ad35 expanded the mean frequency of E7-specific $CD8^+$ T cells whereas a single Ivag booster with Ad26 or Ad35 maintained or further enhanced the mean frequency of $CD8^+$ T cells E7-specific. Together these data indicate that primary $CD8^+$ T cell responses contracted and could be recalled by a secondary immunization. Also, these data indicate that IM immunization is more potent than Ivag immunization for priming and recall of circulating $CD8^+$ T cell responses.

Ivag immunization induces lower type-specific Ad neutralizing antibody titer compared to IM immunization

We evaluated neutralizing antibody titers against Ad26 and Ad35 in plasma samples collected three weeks after the booster immunization. Ivag immunization with either Ad26 or Ad35 induced low to no type-specific neutralizing antibody titers whereas IM immunization with either Ad26 or Ad35 induced high type-specific neutralizing antibody titers (Fig 3C, D). These data suggest that repeated Ivag immunization might be achieved using the same serotype. No detectable cross-neutralizing antibodies were measured upon a single immunization with Ad26 or Ad35, IM or Ivag. Interestingly the neutralizing antibody titers against the type used for priming were lower compared to the neutralizing antibody titers against the type used for booster which might be due to early decrease in vector-specific antibody responses upon a single exposure.

Ad26 and Ad35 transduce intact cervicovaginal mucosa epithelium and are partially inhibited by carrageenan

The use of N-9 in our topical therapeutic vaccination procedure might transiently potentiate HPV reinfection through autoinoculation or from an infected sexual partner [27]. Therefore, we evaluated the ability of Ad vectors to transduce an intact cervicovaginal epithelium or to transduce the cervicovaginal epithelium in the presence of carrageenan, a sulfated polysaccharide that is a potent HPV microbicide [20]. We used Ad vectors expressing luciferase for quantification of *in vivo* transduction and GFP to analyze tropism at the peak of expression as determined in previous experiments (Fig. 1). Although disruption of the cervicovaginal epithelium increased significantly in vivo transduction by Ad26 and Ad35 both types were able to transduce an intact cervicovaginal epithelium. Surprisingly, carrageenan had only a modest effect on Ad26- and Ad35-mediated transduction, respectively, which suggests that glycosaminoglycans play a minor role in cell attachment and infection by these Ad vectors types (Fig. 4A, B). Interestingly, the preferential tropism for the epithelial layers of the cervicovaginal mucosa was conserved across all conditions tested (Fig 4C, D).

Ad26 immunogenicity is retained upon Ivag instillation on intact cervicovaginal epithelium and induces preferentially $CD69^+CD103^+$ tissue resident $CD8^+$ T cells

We evaluated immunogenicity after Ivag priming with Ad26-E6E7fus vector on an intact or disrupted cervicovaginal epithelium either in the absence or presence of carrageenan (Fig. 5). We focused on Ad26 vectors for Ivag immunization because it transduced more efficiently the cervicovaginal epithelium than Ad35 (Fig. 1A, B).

We used 1×10^{10} vp of Ad26-E6E7fus vector for Ivag priming to induce consistent primary $CD8^+$ T cell responses. Mice were pre-treated Ivag with 4% N-9 or with 4% CMC as a control for intact epithelium. Five hours later, Ad26-E6E7fus was diluted in 1% ι-carrageenan or 2% CMC as a control and delivered Ivag. Three weeks after priming, we quantified cervicovaginal HPV16 E7-specific $CD8^+$ T cells by flow cytometry using H2-$D^bE7_{49\text{-}67}$ tetramers (Fig. 5A, row 1). We assessed the expression of CD69 and CD103 markers to determine the tissue resident memory phenotype of E7-tetramer-negative (Fig. 5A row 2) and E7-tetramer-positive (Fig. 5A row 3) cervicovaginal $CD8^+$ T cells.

Interestingly, Ivag priming after N-9 treatment induced the same frequency and number of E7-specific $CD8^+$ T cells with or without carrageenan, consistent with our in vivo transduction data (Fig. 4). Surprisingly, Ivag priming on an intact epithelium induced similar frequency and number of cervicovaginal E7-specific $CD8^+$ T cells to N-9 treated mice despite reduced in vivo transduction efficacy compared to Ivag immunization after N-9 pretreatment (Fig. 5A–C). Carrageenan decreased the induction of E7-specific $CD8^+$ T cell after Ivag priming on an intact epithelium which correlates with lowest in vivo transduction (Fig. 5A–E). All Ivag immunization procedures induced cervicovaginal E7-specific $CD8^+$ T cells with a tissue resident memory phenotype characterized by CD69 and CD103 expression (Fig, 5E). Interestingly, $CD8^+$ tetramer negative fraction was also enriched for Trm cells in all groups immunized Ivag with Ad26 vectors including the group immunized with a Ad26-empty vector but not groups immunized IM with Ad26-E6E7fus or sham immunized (Fig. 5F). These data suggest that Ivag immunization with Ad26 might induce a strong anti-vector $CD8^+$ T cell response. Finally, all Ivag immunization procedures induced circulating E7-specific $CD103^-CD69^-CD8^+$ T cells albeit of lower magnitude than a single IM immunization (Fig. 5D, G).

Ad26-E6E7fus Ivag booster immunization on intact cervicovaginal epithelium induces a broad polyfunctional $CD8^+$ T cells response against the HPV16 E6 and E7 antigens

Next, we evaluated the $CD8^+$ T cells cytokine response after IM prime with Ad35-E6E7fus followed by booster Ivag immunization with Ad26-E6E7fus vector onto intact or disrupted cervicovaginal epithelium (+/− N-9) with or without the addition of carrageenan (+/− Carr). As immunization controls some mice that were primed IM with Ad35-E6E7fus were then Ivag immunized with Ad26-mock pretreated with N-9 (mock) or treated with N-9 only (sham); or primed IM with Ad35-mock and immunized later Ivag with Ad26-E6E7fus with N-9 (Ad26-E6E7); or primed IM with PBS (sham) and later treated with N-9 only (sham). Spleen and cervicovaginal single cell suspensions were incubated with E6 or E7 overlapping peptide mix, $E7_{49\text{-}67}$ peptide, PMA/ionomycin as a positive control or in medium only as negative control. IFN-γ production by $CD8^+$ T cells was measured by intracellular cytokine staining as shown in Figure 6A.

E7-specific IFN-γ production by $CD8^+$ T cells in the spleen was observed in all groups primed IM with Ad35-E6E7fus (Fig. 6B) but was higher in groups receiving a booster Ivag immunization compared to group not receiving a booster immunization. Interestingly, an intact or disrupted epithelium with or without carrageenan did not impact the recall of systemic $CD8^+$ T cell responses after Ivag immunization (Fig. 6B). Importantly, in vitro stimulation of spleen cells with the minimal immunodominant epitope $E7_{49\text{-}67}$ peptide induced similar frequency of IFN-γ^+CD8^+ T cells compared to the E7 peptides mix, which suggests that most E7-specific $CD8^+$ T cells reacted to the $E7_{49\text{-}67}$ immunodominant epitope. E6-responding IFN-γ^+CD8^+ T cells were detected in the spleen although at lower level than E7-specific IFN-γ^+CD8^+ T cells. Most spleen HPV-specific $CD8^+$ T cells induced after IM Ad35-E6E7fus immunization were polyfunctional producing simultaneously IFN-γ and TNF-α (Fig. 6C) and were expanded by Ivag booster immunization with Ad26-E6E7fus.

Due to limited number of cells extracted from the cervicovaginal mucosa, we performed each in vitro stimulation condition on pooled single cell suspensions of groups of 5 mice. All groups primed IM with Ad35-E6E7fus and boosted Ivag with Ad26-E6E7fus displayed high frequency of polyfunctional $CD8^+$ T cells producing IFN-γ and TNF-α simultaneously in E6 and E7 overlapping peptide mix in vitro stimulation (Fig. 6D). Ivag booster immunization with Ad26-mock vector did not redirect circulating E6/E7-specific $CD8^+$ T cells induced by IM prime with Ad35-E6E7fus to the vaginal tract (Fig. 6D). However, all groups receiving Ivag Ad26-E6E7fus or Ad26-mock displayed a high frequency of polyfunctional $CD8^+$ T cells upon stimulation with PMA/ionomycin compared to sham treated groups. These data strongly suggest that Ivag immunization with Ad vectors triggers vector-specific $CD8^+$ T cell responses.

Analysis of cervicovaginal $CD4^+$ T cell responses after IM/Ivag prime-boost immunization with Ad35 and Ad26 vectors

Using E6 and E7 15mer overlapping peptides for in vitro stimulation, we did not detect E6 or E7-specific $CD4^+$ T cells producing IFN-γ or TNF-α (Fig S2 and data not shown). All groups boosted Ivag with Ad26-E6E7fus showed infiltration of the cervicovaginal mucosa by $CD4^+$ T cells (Fig S2B). Most infiltrating $CD4^+$ T cells expressed CD69 but not CD103 suggesting a submucosal resident phenotype (Fig S2C). Interestingly, in all groups immunized Ivag with Ad26-E6E7fus, PMA/ionomycin restimulation induced higher $CD4^+$ T cells IFN-γ and TNF-α production than in sham-treated groups (Fig S2D). Increased $CD4^+$ T cells infiltration and cytokine production was also observed in mice boosted Ivag with Ad26-mock compared to sham-treated suggesting induction of adenovirus-specific $CD4^+$ T cells (Fig S2). Next, we assessed the role of $CD4^+$ T cells in the induction of $CD8^+$ T cells after Ad26-E6E7fus Ivag immunization (Fig S3A–B). Antibody mediated CD4-depletion prevented the induction of $tetramer^+CD8^+$ T cell responses in blood and cervicovaginal mucosa and suppressed the induction of E7-specific IFN-γ^+TNF-α^+ $CD8^+$ T cells in spleen (Fig S3C–E).

Discussion

Ad26 and Ad35 were developed as alternative types to Ad5 to circumvent vector-specific neutralizing antibodies for prime-boost immunization. Clinical grade Ad26 and Ad35 have been mostly utilized for systemic delivery, with a few exceptions for intranasal delivery. Preclinical evaluation of Ad vectors indicates that the quality of the memory $CD8^+$ T cells elicited after IM immunization varies depending on the Ad serotype[28]. These differences in immunogenicity might result from altered antigen expression due to differences in cellular tropism and receptor usage or altered modulation of innate cytokine responses between Ad serotypes[29].

In this study, we show that Ad26 and Ad35 vectors transduce the intact mouse cervicovaginal epithelium, whereas HPV pseudovirions, used for Ivag immunization, require epithelium permeabilization or disruption to infect basal cells of pluristratified epithelia[27]. Such difference might be due to the expression of Ad specific receptors and attachment factors at the apical side of epithelial cells. Also, Ad fiber proteins have been shown to

increase airway epithelium permeability through binding to the Coxsackievirus and Adenovirus Receptor (CAR), resulting in disruption of tight junctions and spread of viral particles to the lumen[30]. A similar mechanism might facilitate transduction of the epithelial cells upon Ivag instillation. Carrageenan inhibition experiments indicate that glycosaminoglycans may play a limited role in cell attachment and infection by Ad26 and Ad35 vectors, consistent with previous studies with Ad2 and Ad5[31]. Ad vectors tropism might differ between human and mice due to differential expression of receptors such as CAR or CD46 and might constitute a limitation to our preclinical evaluation [32]. However, because mucosal epithelia are the natural sites of infection by human Ad, it is expected that Ad vectors should retain their strong ability to transduce the cervicovaginal epithelium in women.

Few preclinical studies have evaluated Ivag delivery of a vaccine for treatment of HPV-induced neoplasia. Ongoing clinical trials for therapeutic treatment of CIN are introducing the use of topical TLR agonists such as Imiquimod to subvert the immune suppressive environment, promote immunogenic cell death and recruit HPV-specific T cells[33]. To date, impressive clinical response has been observed in a large clinical trial involving multiple rounds of intralesional injection of an MVA vector expressing bovine papillomavirus E2 protein[34]. Ongoing clinical trials are combining vaccination with topical Imiquimod to promote trafficking of antigen-specific T cells to the lesion (NCT00788164, NCT00988559). Together these studies underscore the potential of therapeutic interventions targeting the lesion site.

Our results challenge the notion that the cervicovaginal mucosa is poorly immunogenic. We provide evidence for the first time that Ivag vaccination with Ad26-E6E7fus or Ad35-E6E7fus increases trafficking of HPV-specific $CD8^+$ T cells to the cervicovaginal mucosa. Our results extend previous studies showing immunogenicity and protection after Ivag delivery of Ad5 expressing HIV-1 GAG or HSV-2 gB antigens in preclinical infection models[25, 35]. Our immunization strategy provides a way to amplify local $CD8^+$ T cell responses by IM priming immunization followed by Ivag boosting. Local antigen presentation of E6 and E7 antigens might explain the preferential induction of cervicovaginal Trm $CD8^+$ T cells compared to sole IM immunization. Such presentation might trigger the recruitment of preexisting circulating memory $CD8^+$ T cells, as demonstrated in the context of genital viral infection[36]. Local proliferation might also explain the expansion of HPV-specific $CD8^+$ T cells as shown after Ivag priming or boosting with other viral vectors[19, 37].

The fact that primary $CD8^+$ T cell responses depend on the presence of $CD4^+$ T cells after Ivag immunization with Ad26 vectors is concordant with the requirement of $CD4^+$ T cell help in the formation and maintenance of memory $CD8^+$ T cells after IM immunization[38]. We did not detect $CD4^+$ T cell responses against the E6E7fus antigen after restimulation with E6 and E7 15mer peptide mixes which should have allowed to detect most $CD4^+$ T cells specific of classical MHC-II restricted peptides[39]. Therefore, it is possible that $CD4^+$ T helper cells instead react to adenoviral antigens. $CD4^+$ T cell responses against HPV16 E6 and E7 antigens are known to be weak in the C57BL/6 strain of mice, which harbors only one MHC-II molecule, and so may be suboptimal for induction of CD8+ T cell responses[40].

We speculate that such E6- and E7-specific $CD4^+$ T cell responses might be easier to elicit in outbred human populations with higher diversity and polymorphisms of MHC-II molecules.

Our study provides evidence that Ivag vaccination with Ad26 or Ad35 vectors induces high number of $CD8^+$ Trm cells in the cervicovaginal mucosa. $CD8^+$ Trm are excluded from the circulation which prevents their monitoring in blood samples in the context of infections or vaccination. Seminal studies in humans have described the anatomical distribution of memory $CD8^+$ T cells including Trm and validated CD103 and CD69 as markers of $CD8^+$ Trm at body surfaces [41, 42]. However, the precise contribution of $CD8^+$ Trm to spontaneous clearance of HPV persistent infection or regression of CIN has not yet been studied. Local amplification of $CD8^+$ Trm might overcome early immune escape mechanisms and shift the ratio of effector CTL/target cells in favor of improved clearance. Finally, local induction of vector-specific $CD8^+$ Trm might trigger bystander activation of $CD8^+$ Trm and could lead to tissue conditioning to an anti-viral state and promote virological clearance or lesion regression. Such bystander effects have been described with viral vectors or peptides against CIN or viral infections[34, 43–45].

Accumulating evidence suggests that, early upon infection, lesions evolve toward an immunosuppressive state as shown by up regulation of PD-L1, exclusion of T cells from infiltrating the lesion, and/or down regulation of MHC molecules. The current syngeneic mouse orthotropic models for HPV-associated cancer are associated with rapid tumor growth, do not recapitulate the slow progression of CIN[46, 47] and prevent the evaluation of our prime/boost vaccination regimen. The primary goal of this study was to evaluate immunogenicity of Ad vectors given Ivag in a prime/boost vaccination regimen, in particular $CD8^+$ T cells trafficking to the cervicovaginal epithelium. IM/Ivag immunization was by far the best combination to induce simultaneous production of IFN-γ and TNF-α by E6- and E7-specific $CD8^+$ T cells and was higher in the cervicovaginal mucosa compared to secondary lymphoid organs. Production of both cytokines is the best indication that the cervicovaginal $CD8^+$ T cells are functional and endowed with potentially anti-viral and anti-tumoral activities[48]. In addition to direct tumoricidal activities, local production of IFN-γ and TNF-α could increase expression of MHC molecules by transformed cells and render them susceptible to $CD8^+$ T cells-mediated cytolysis.

The feasibility of translating our vaccination strategy to treat cervical and vaginal intraepithelial neoplasia and persistent high-risk HPV infection in women should be evaluated. Several clinical trials involving intravaginal delivery of drugs or microbicides support the conclusion that self-administration of gel-based vaginal vaccine might be well accepted and tolerated[49, 50]. We believe that a self-administered adenoviral vaccine could be a critical component of a single clinic visit screen-and-treat public health program to lower cervical cancer rates in mid-adult women in low resource settings. In this scenario, a positive result in a point-of-care HPV test of a cervicovaginal sample would trigger an Ad IM prime vaccination in positive women followed several weeks later by an in-home Ad Ivag booster immunization. This strategy might induce the accumulation of intralesional E6- and E7-specific $CD8^+$ Trms, overcome local immune suppression in early and advanced lesions and promote high rates of virological clearance and/or CIN regression.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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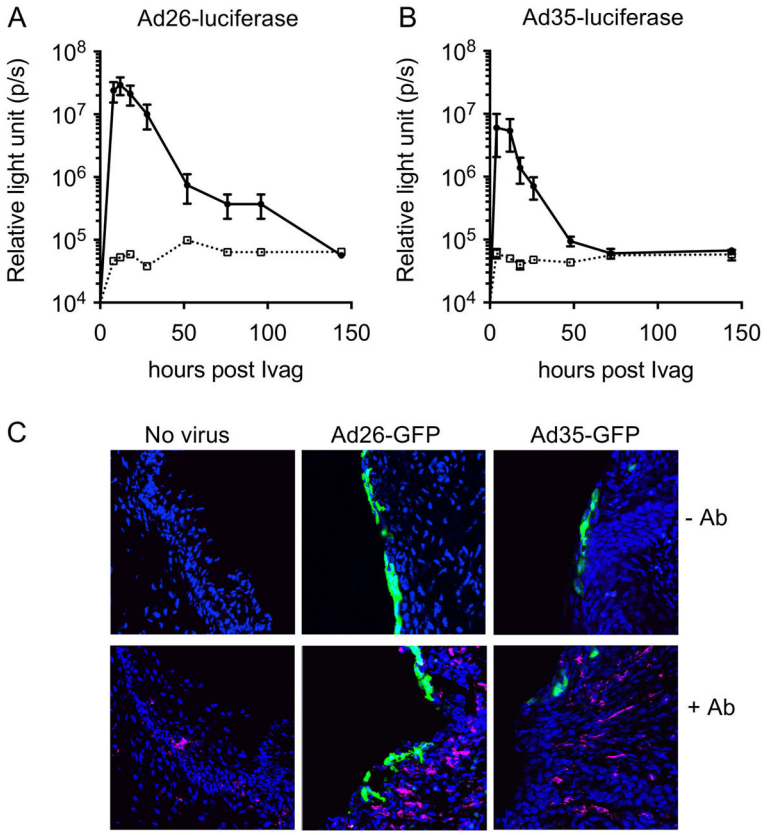

Figure 1.

Intravaginal instillation of Ad vectors type 26 and 35 allows efficient gene delivery to the cervicovaginal mucosa epithelium. Depoprovera-treated mice were inoculated Ivag with N-9 followed 5 hours later with Ad vectors (2.5×10^9 vp) formulated in 2% carboxymethyl cellulose. Longitudinal in vivo bioluminescence imaging of the genital region of mice inoculated Ivag of with Ad26 (A, solid line) and Ad35 (B, solid line) vectors expressing firefly luciferase or without virus (A–B, dashed line). Data are expressed as mean relative light unit +/− SEM (A–B). For each time point (8hr to 144hr), 20ul of of 15mg/ml D-luciferin was instilled Ivag 3 min prior to imaging. Confocal microscopy imaging of GFP expression in the cervicovaginal mucosa epithelium after Ivag instillation of Ad26 and A35 vectors expressing GFP and immunofluorescent staining with (+Ab) or without (-Ab) Alexa-647 conjugated anti MHC-II antibody (C).

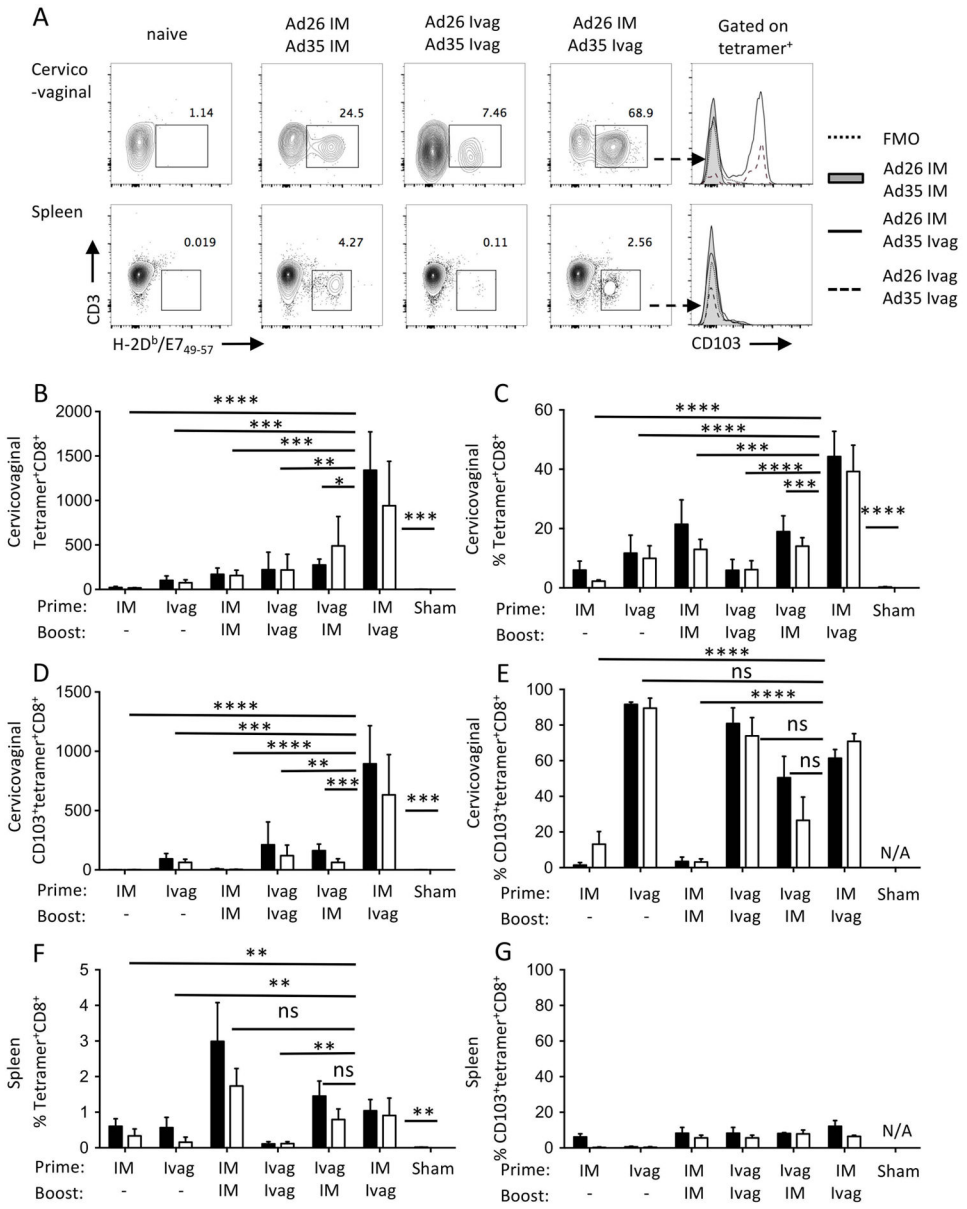

Figure 2.

Prime boost immunization via heterologous routes with Ad vectors affects differentially the induction of systemic and cervicovaginal HPV-specific $CD8^+$ T cell responses. Depoprovera-treated mice were immunized Ivag following N-9 pretreatment or IM with Ad vectors (2.5×10^9 vp) expressing a fusion protein of the HPV16 E6 and E7 oncoproteins. Mice (n= 5/group) were immunized sequentially one month apart with Ad26 for the prime and Ad35 for the boost (black histogram bars) and conversely with Ad35 for prime and Ad26 for boost (open histogram bars). Cervicovaginal (A–E) and spleen (A, F–G) cell suspensions were analyzed by flow cytometry for the presence of two weeks after immunization. HPV16 E7-specific $CD8^+$ T cell responses are expressed as total number per organ (B) and percentage of $CD8^+$ T cells (C and F). CD103 expression by HPV16 E7-specific $CD8^+$ T cells is expressed as total number per organ (D) and percentage (E and G).

Data are expressed as mean percentage or mean number of cells per organ ±SEM (N/A, not applicable). One-way analysis of variance and multiple comparisons to the sham-treated group (Kruskal-Wallis H test) was performed on groups based on the route of immunization.

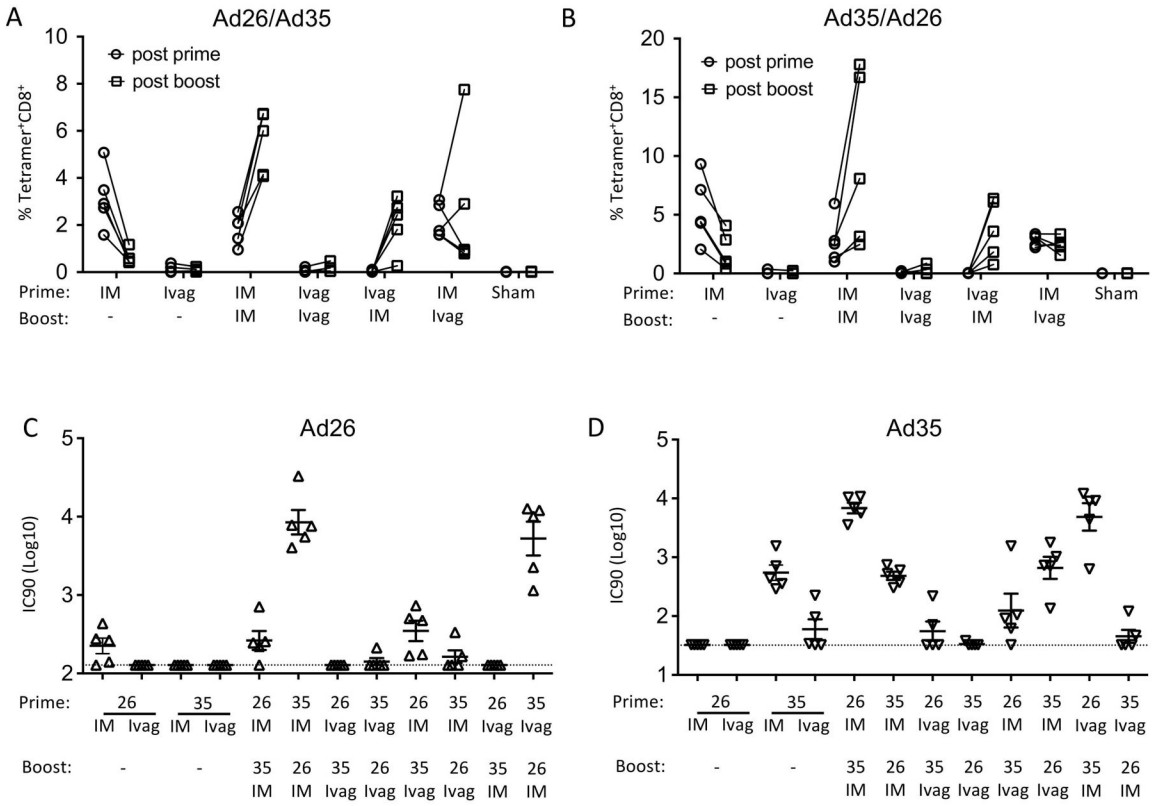

Figure 3.

The route of immunization with Ad vectors affects differentially the induction of circulating HPV16 E7-specific $CD8^+$ T cells and adenovirus type-specific neutralizing antibodies. Depoprovera-treated mice were immunized Ivag following N9 pretreatment or IM with Ad vectors (2.5×10^9 vp) expressing a fusion protein of the HPV16 E6 and E7 oncoproteins. Mice were immunized sequentially one month apart with Ad26 for the prime and Ad35 for the boost (A) and conversely (B). All routes were tested alone by single immunization with Ad26 (A) or Ad35 (B). Blood samples were analyzed by H2-D^b/$E7_{49\text{-}57}$ tetramer staining for the presence of HPV16 E7-specific CD8+ T cells in blood samples two weeks after immunization. Symbols connected by a line represent individual mice with open circles and open squares corresponding to post prime and post boost analysis, respectively (A, B). Adenovirus neutralizing antibody titers were measured in serum samples two weeks after the final immunization (C, D). Titers are expressed as mean (horizontal bar) and individual mice (symbols) for Ad26 (C, open triangle) and Ad35 (D, inverted open triangle).

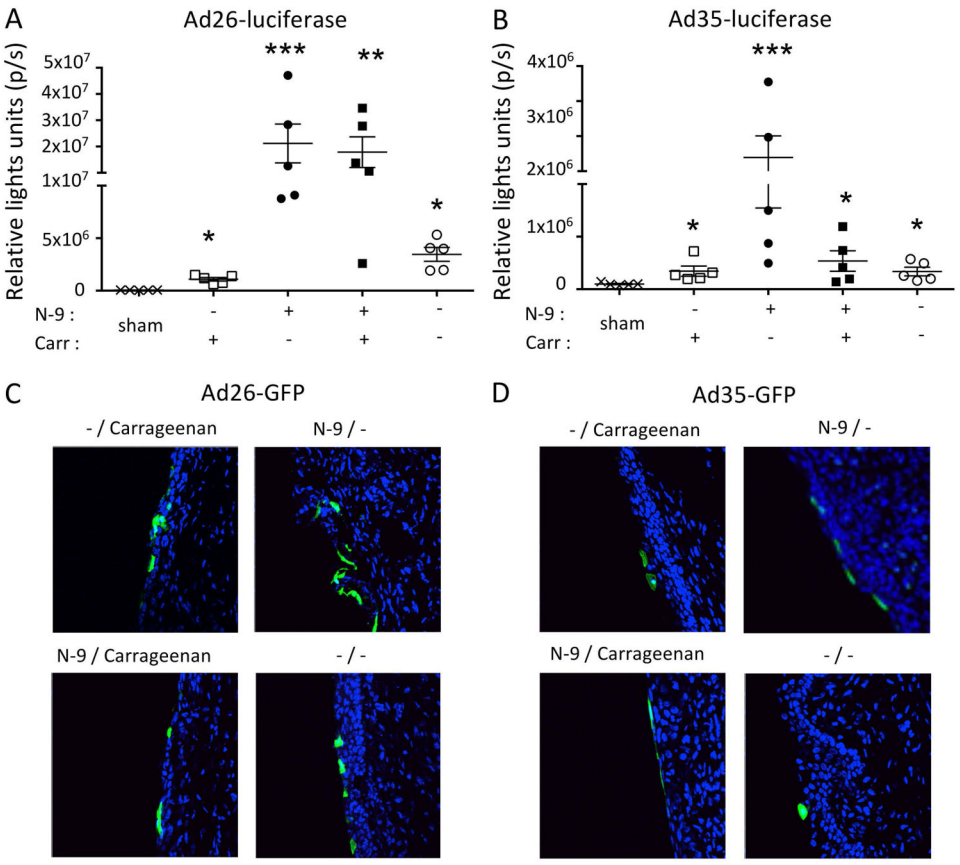

Figure 4.

Effect of N-9 and ι-carrageenan on Ad vector type 26 and 35 transduction of the cervicovaginal mucosa. Depoprovera-treated mice (n=5/group) were inoculated Ivag with or without N-9 followed 5 hours later with Ad vectors (2.5×10^9 vp) given in CMC or i-carrageenan. Longitudinal in vivo bioluminescence imaging of the genital region of mice inoculated Ivag of with Ad26 (A) and Ad35 (B) vectors expressing firefly luciferase. Data are expressed as mean +/− SEM (A–B). For each time point (18hrs), 20ul of of 15mg/ml D-luciferin was instilled Ivag 3min prior to imaging. Confocal microscopy imaging of GFP expression in the cervicovaginal mucosa epithelium after Ivag instillation of Ad26 (C) and A35 (D) vectors expressing GFP. One-way analysis of variance and multiple comparisons (Kruskal-Wallis H test) was performed to determine statistical significance.

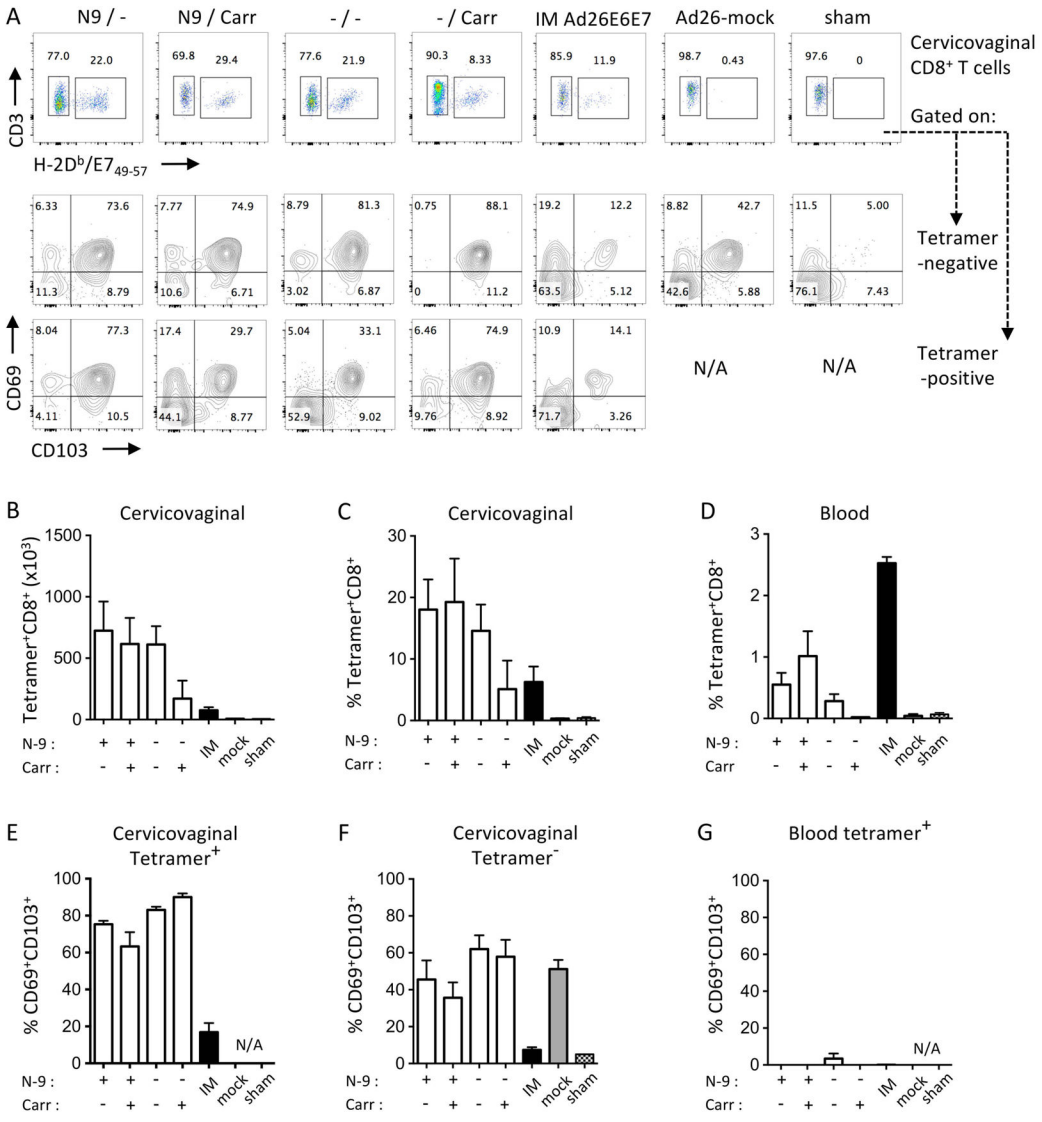

Figure 5.

Effect of N-9 and ι-carrageenan on Ad26 immunogenicity after Ivag priming. Depoprovera-treated mice (n=5/group) were inoculated Ivag with or without N-9 followed 5 hours later with Ad26-E6E7fus (1.0×10^{10} vp) delivered in CMC or ι-carrageenan. Additional groups, IM Ad26-E6E7fus (IM), Ivag Ad26-mock with N-9 and no carrageenan (mock) and Ivag N-9 without carrageenan (sham) were used as control. Cervicovaginal (A–C, E–F) and blood samples (D–G) were analyzed by H2-D^b/$E7_{49-57}$ tetramer staining for the presence of HPV16 E7-specific $CD8^+$ T cells three weeks after immunization. Representative FACS plot for H2-D^b/$E7_{49-57}$ tetramer staining on cervicovaginal $CD8^+$ T cells (A, row1) and for CD69 and CD103 expression by tetramer negative (A, row2) and tetramer-positive (A, row3). Data are expressed as mean percentage or mean number of cells per organ ± SEM (N/A, not applicable).

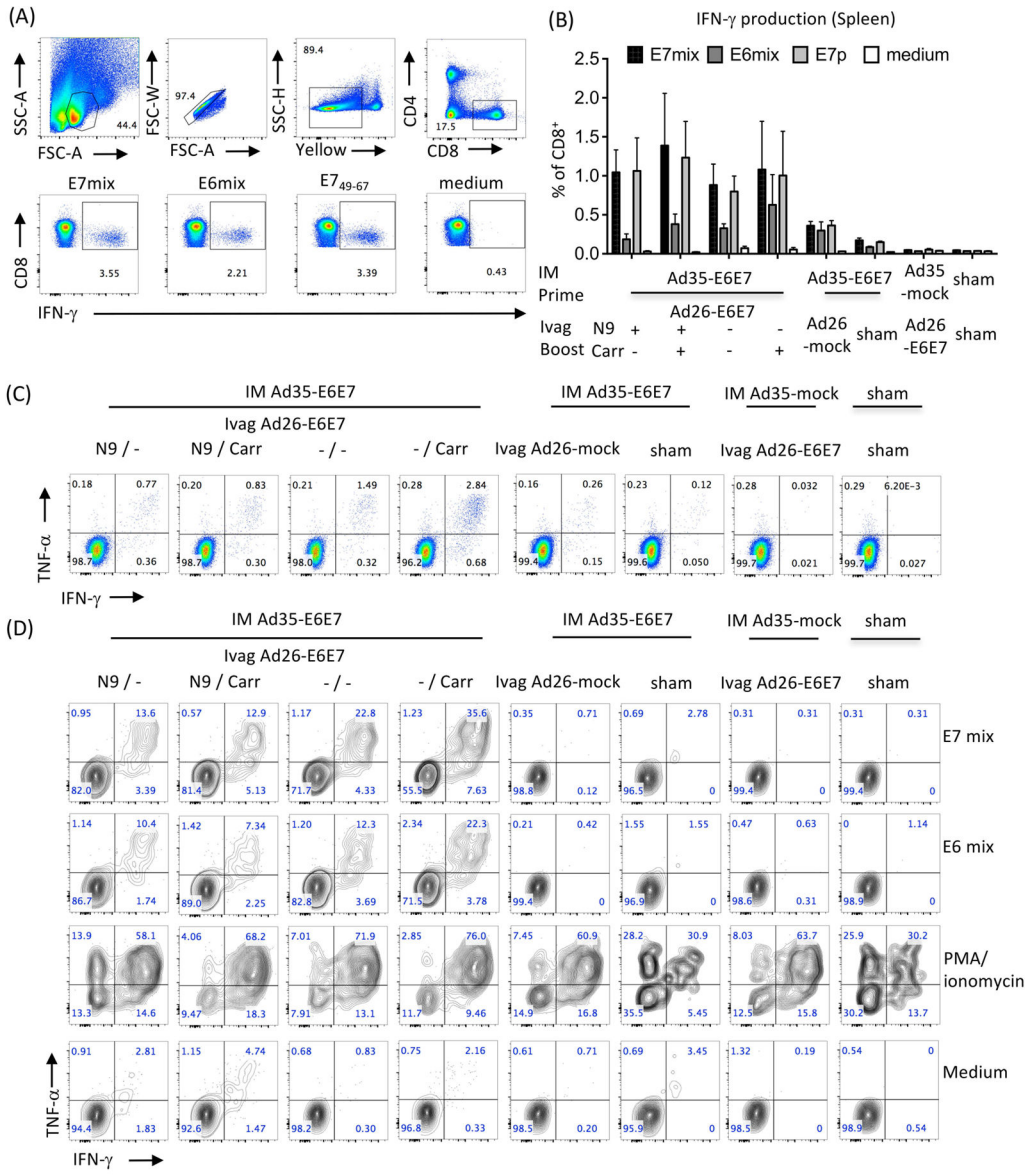

Figure 6.

Effect of N-9 and ι-carrageenan on the induction of cervicovaginal and systemic polyfunctional CD8$^+$ T cells after Ivag booster immunization with Ad26-E6E7fus. Mice (n=5/group) were immunized IM with Ad35-E6E7fus (1.0×10^{10} vp), Ad35-mock (1.0×10^{10} vp) or PBS as control. One month later, Depoprovera-treated mice were inoculated Ivag with or without N-9 (+/−) treatment followed 5 hours later with Ad26-E6E7fus (1.0×10^{10} vp) delivered in CMC or ι-carrageenan (+/−). As controls, some groups primed IM with Ad35-E6E7fus were immunized later Ivag with Ad26-mock (1.0×10^{10} vp) with N-9 (mock) or treated with N-9 only (sham); primed IM with Ad35-mock were immunized one month later Ivag with Ad26-E6E7fus following treatment with N-9 (Ad26-E6E7); additional controls include mice primed IM with PBS (sham) and treated one month later with with N-9 only (sham). Three weeks after booster immunization, cytokine production by spleen (A, B) and cervicovaginal (C, D) CD8+ T cells was assessed by intracellular cytokine

staining after in vitro stimulation with overlapping peptide mixes of E7 (black bars in B) or E6 (dark grey bars in B) $E7_{49-67}$ (light grey bars in B), medium only (white bars in B) or PMA/ionomycin. FACS plot showing the gating strategy and analysis of IFN-g production by splenic $CD8^+$ T cells (A, B). FACS plot presentative of IFN-γ and TNF-α production by splenic (individual samples) and cervicovaginal (pooled samples) $CD8^+$ T cells (C, D). Data are expressed as mean percentage of cells ± SEM (5 mice per group).