npj Vaccines

https://doi.org/10.1038/s41541-026-01467-z

Article in Press

# Intranasal boosting enhances genital tract T cell immunity following ChAdOx1/MVA hrHPV therapeutic vaccination

Received: 14 January 2026

Accepted: 16 April 2026

Cite this article as: Hancock, G., Evans, T., López-Camacho, C. *et al.* Intranasal boosting enhances genital tract T cell immunity following ChAdOx1/MVA hrHPV therapeutic vaccination. *npj Vaccines* (2026). https://doi.org/10.1038/s41541-026-01467-z

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**Title:** Intranasal boosting enhances genital tract T cell immunity following ChAdOx1/MVA hrHPV therapeutic vaccination

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**Abstract**

High risk human papillomaviruses (hrHPV) infect mucosal epithelia and are the causative agents of cervical cancer. Prophylactic vaccines protect against disease caused by several, but not all hrHPV types. Furthermore, prophylactic vaccines are not widely available to populations with the greatest disease burden and have no therapeutic effect in previously infected individuals. Therapeutic vaccines that enhance host immunity in individuals with persistent hrHPV infections represents a promising strategy to support cervical cancer control. However, efficacy of a therapeutic vaccine depends not only on antigen selection and delivery system, but also on the

route of immunisation. Because cervical intraepithelial lesions are confined to the genital mucosa, an effective therapeutic vaccine may need to elicit immune effector cells at the site of disease. Here we study the effect of immunisation route on vaccine immunogenicity using previously described ChAdOx1- and MVA-vectored hrHPV therapeutic vaccines encoding a multigenotype insert. In addition, we used GFP-expressing viral-vectored vaccines to monitor vaccine uptake in the cervix by real-time in vivo imaging. Among all regimens tested, the intramuscular prime–intranasal boost induced the highest magnitude of E6-specific CD8+ T cells in both the spleen and cervicovaginal compartment, and also generated the greatest frequency of cervicovaginal CD8+ $T_{RM}$ cells.

**Keywords:** HPV, Cervical cancer, vaccine administration, therapeutic vaccine, immunotherapy



## Introduction

Cervical cancer, the consequence of persistent infection by high-risk type human papillomaviruses (hrHPV) [1], is one of the deadliest and most preventable cancers. It is the fourth most common cancer among women worldwide, with approximately 660,000 new cases and 350,000 deaths annually. The highest incidence and mortality rates occur in low- and middle-income countries (LMICs), where it is the second most common cancer among women[2–4]. The majority of hrHPV infections are transient and subclinical due to rapid immune clearance. However, persistent hrHPV infection can lead to the development of precancerous lesions (cervical intraepithelial neoplasia; CIN). Untreated high-grade lesions (CIN2, CIN3) can progress to invasive cervical carcinomas although this may take 10-20 years. Licensed prophylactic vaccines are highly effective in preventing infection with HPV16 and HPV18, which together cause two-thirds of cervical cancers

worldwide and the introduction of the nonavalent vaccine has extended coverage to five more high-risk types [5]. However they offer no therapeutic benefit in individuals previously infected with hrHPV and provide only partial protection against infection from other oncogenic HPV types not covered by the vaccines [6]. Millions of women worldwide remain at risk of developing cervical cancer because of both limited access to HPV vaccination and cervical screening programs. Effective therapeutic vaccines targeting hrHPV infection could therefore have an immediate and substantial impact on the mortality and morbidity of hrHPV associated malignancies and CIN. As with prophylactic vaccines, type specificity remains an important consideration for therapeutic vaccine design.

CIN lesions are mostly restricted to the genital mucosa, a unique immunological environment in which immune effector responses must be balanced against immunological tolerance. Regression of these lesions is therefore likely to require vaccine induced, cell-mediated immune responses at the local mucosal site of the disease. Consistent with this, early infiltration of cytotoxic effector T cells in low grade genital lesions has been associated with protection against tumour progression [7]. The route of administration could significantly influence the magnitude and localisation of the immune response, and it is therefore an important determinant of vaccine efficacy. Most licensed vaccines are delivered by intramuscular (IM) or subcutaneous (SC) routes, however, these routes elicit limited mucosal cell-mediated immunity, which may partly explain the modest efficacy observed in clinical trials of systemically administered therapeutic HPV vaccines to date [8–10]. In a Phase 2b trial of VGX-3100, a therapeutic DNA vaccine encoding HPV16 and HPV18 E6 and E7, delivered intramuscularly (IM) by electroporation, histopathological regression occurred in 49.5% of vaccine recipients compared with 30.6% of placebo recipients [8]. We recently published

the outcome data of a clinical trial using a heterologous, multi-antigenic HPV two-component immunotherapy delivered IM, showing that the regimen was immunogenic but did not result in a statistically significant clearance of either hrHPV or the associated cervical lesions [9]. Alternative routes of administration, such as intranasal (IN) or intracervical (ICV) delivery, as well as prime-boost combinations that involve both mucosal and systemic delivery, may be critical for directing vaccine-induced immune responses to the cervicovaginal mucosa and thereby enhancing local immunity and therapeutic efficacy. Viral vectors have been investigated as platforms for mucosal vaccine delivery, including in the context of COVID-19, where adenoviral-vectored vaccines delivered intranasally or by inhalation have received regulatory approval in some countries [11,12]. More specifically, viral vector-based mucosal vaccination has been shown to direct antigen-specific T cell responses to the female genital tract[13–15]. In a preclinical study, Çuburu et al., demonstrated that intramuscular priming with Ad26 or Ad35 followed by intravaginal adenoviral boosting elicited robust antigen-specific CD8+ T cell responses in the genital mucosa [16]. Although fewer studies have evaluated modified vaccinia Ankara (MVA) via mucosal routes, preclinical investigations indicate that mucosal delivery of MVA can induce local and systemic T cell responses, supporting its potential utility in this context [17,18].

The heterologous Adenovirus/MVA prime–boost platform is well established for inducing high magnitude antigen-specific T cell responses following systemic delivery [19]. We have previously shown that HPV-specific CD8+ T cells were present in the cervix following systemic prime-boost administration of ChAdOx1-5GHPV3 and MVA-5GHPV3 (subsequently renamed VTP-200 by Barinthus Biotherapeutics), and that their frequency increased over time, consistent with continued trafficking of T cells to the cervix [20]. However, whether alternative routes of vaccination could

further enhance the magnitude of T cell response at the site of disease remains unknown. To address this, we evaluated the immunogenicity of ChAdOx1-5GHPV3 prime followed by MVA-5GHPV3 boost using homologous (IM/IM, IN/IN, ICV/ICV) and heterologous (IM/IN, IM/ICV) vaccination regimens in a murine model. Our study specifically investigates how mucosal and systemic vaccine delivery modulates mucosal and systemic immune responses, with the aim of optimising prime-boost vaccination strategies for the treatment of hrHPV-associated precancerous lesions and malignancies.



## Methods

*Vaccine vectors and Mouse immunisations*

We previously designed the synthetic gene '5GHPV3′ by selecting conserved regions from each of the six hrHPV early proteins and generating consensus sequences to represent five hrHPV genotypes [20]. 5GHPV3 was cloned into ChAdOx1 and MVA vectors. ChAdOx1-GFP and MVA-GFP were used to monitor the efficiency of intracervical vaccination. Six-week-old female C57BL/6 mice were purchased from Harlan, UK. Mouse care and experimental procedures were carried out in accordance with the UK Animals (Scientific Procedures) Act under Project Licence PP8738432 and were approved by the University of Oxford Animal Care and Ethical Review Committee. Groups of six C57BL/6 female mice were primed by systemic (intramuscular) or mucosal (intracervical and Intranasal) administration of $1x10^8$ IU/ml ChAdOx1-5GHPV3 and then boosted two weeks later (d14) with $1x10^7$ PFU MVA-5GHPV3 by a homologous or heterologous vaccination route (IM, ICV or IN) (Table 1). For vaccinations performed via the intramuscular

(IM) route, a final volume of 50 µl was injected into the tibialis anterior muscle of each animal (25 µl per anterior muscle). For intranasal (IN) vaccine administration we used the same vaccine formulation as for the intramuscular injection. Mice were anesthetised and the vaccine was administered dropwise into the nasal cavity (50 µl). For intracervical (ICV) vaccine delivery, we adapted the method of transcervical embryo transfer (TCET) [21] a nonsurgical procedure involving insertion of a speculum into the vagina and passage of a flexible catheter through the cervix to deposit vaccine (15 µl of ChAdOx1-5GHPV3 or MVA-5GHPV3) into the cervical canal under anesthesia (TCET, Elim Springs Biotech). All viruses were resuspended in endotoxin-free PBS for immunization. Mice were culled on d14, d21 and d28 and female reproductive tracts and spleens were harvested.

*In vivo and ex vivo imaging*

To evaluate the efficiency of the modified TCET technique for intracervical vaccination, GFP expression was assessed following intracervical instillation of ChAdOx1-GFP and MVA-GFP. CD1 mice were chosen for imaging experiments due to the light absorbance properties of black fur in C57BL/6 mice, which could interfere with fluorescence detection. GFP expression was monitored using the Xenogen IVIS Spectrum Imaging System (PerkinElmer) at 24 hours following intracervical instillation of ChAdOx1 and MVA vectors both expressing GFP. For in vivo imaging, mice were anesthetised with isoflurane and shaved from the neck to the lower torso in order to allow for optimal visualisation of fluorescence. Animals were positioned supine in the IVIS Spectrum Imaging System and imaged according to the manufacturer's guidelines. For ex-vivo imaging of cervices, mice were euthanised at 24 hours post-intracervical instillation of ChAdOx1-

GFP and MVA-GFP. Female reproductive tracts (FRT) were carefully excised and placed on sterile 10-cm culture dishes for imaging. Imaging parameters were optimised with an excitation wavelength of 465nm and emission at 520nm, using a calibrated field of view to accommodate multiple subjects. Images were acquired with the Living Image software (v 4.3.1.0) for data analysis, ensuring signal levels exceeded noise thresholds and remained within the CCD dynamic range.

Whole-body imaging revealed no detectable GFP signal above that observed in naïve control mice. Imaging of the dissected FRT following background subtraction using signals from naïve controls, showed robust GFP expression (Supplementary Figure 1), confirming effective delivery via intracervical instillation and validating the technique for subsequent use in vaccination studies with ChAdOx1-5GHPV3 and MVA-5GHPV3.



*Sample processing*

Female reproductive tracts and spleens were harvested from mice at d0 (naïve mice) and d14, d21, d28 post-prime vaccination (vaccinated mice). Excess adipose and unwanted tissue were removed from the isolated female reproductive tracts. The ovaries and fallopian tubes were excised and discarded, leaving the cervix and vaginal regions. The cervicovaginal tract was washed and then digested by incubation with 1 mg/ml collagenase type VIII (Sigma) on a MACSmix rotator at 37 °C for 30 minutes. The tissue was further disrupted using a sterile 70 μm cell strainer. To inhibit further digestion, 10 ml of R15 medium (RPMI supplemented with 15% foetal calf serum, 5ml penicillin/streptomycin and 5 ml L-glutamine) was added to each filtered cell suspension and

samples were placed on ice. The remaining tissue was subjected to two further rounds of digestion following the same protocol. The cervicovaginal cells were resuspended in R10 complete medium (RPMI supplemented with 10% foetal calf serum, 5ml penicillin/streptomycin and 5ml L-glutamine) and rested overnight. Spleens were harvested and single cell suspensions were obtained by passage through a 70 µm cell strainer. Splenocytes were incubated in ammonium-chloride-potassium (ACK) lysis buffer to lyse red blood cells, washed and resuspended in R10 complete medium. After resting overnight at 37 °C, 5% $CO_2$, splenocytes were counted manually to assess cell viability by trypan blue dye exclusion.

*Pentamer staining*

Splenocytes and cervicovaginal cells were first stained at RT for 10 minutes with a PE-conjugated H-2Kb E6 (EVYNFAYTDL) pentamer (Proimmune). This was followed by staining with Zombie-NIR viability dye at RT for 20 minutes to identify live cells. Subsequently, cells were stained with a panel of fluorochrome-conjugated antibodies: BUV615 Anti-CD3, BUV395 anti-CD8, BUV805 anti-CD69, BV421 Anti-CD49d, BV510 anti-CD45, BV650 anti-CD4, BV750 anti-CD103, AF488 anti-CD29, PerCPcy5.5 anti-CD44, PE-Vio770 anti-CD62L and APC anti-CD127 for 20 min at 4 °C. Following staining, samples were acquired using a Cytek Aurora spectral flow cytometer.

*Intracellular cytokine staining*

Splenocytes were resuspend at 10x10^6/ml in R10 and BV421-CD107, GolgiStop (1.6 µl/1000 µl) and GolgiPlug (2 µl/1000 µl) were added. E6 peptides at 2µg/ml were added to test wells, R10 with DMSO to negative wells and PHA to positive wells. Cells were incubated for 6 hours at 37+/-1°C / 5% CO2 in the dark and then held at 2-8°C for up to 18 hours. The next day, cells were stained with zombie-NIR, for 20 minutes, followed by surface staining (BUV395-CD8, BUV615-CD3, BV510-CD45 and BV650-CD4) for 20 min at 4°C protected from direct light. Next, cells were washed and 100 µL of Cytofix-Cytoperm was added and cells incubated for 20 min at 4°C protected from direct light. Intracellular cytokine staining was performed with antibodies against TNF-α (FITC), IL-2 (PE), and IFN-γ (PE-Cy7). Cells were washed twice with BD Perm/Wash buffer, resuspended, and acquired using the Cytek Aurora flow cytometer.

*Statistical analysis*

Graphs and statistical analysis were performed using GraphPad Prism version 9.00. One-way analysis of variance (ANOVA), followed by Tukey's multiple comparisons test or two-way ANOVA followed by Sidak's multiple comparison were performed. Results are indicated as follows: *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, ****$p \leq 0.0001$. SPICE 6 was used to determine statistically significant differences in T cell polyfunctionality using permutation tests.

## Results

### Systemic prime vaccination with a mucosal boost elicits high magnitude E6-specific CD8+ T cell responses across systemic and cervicovaginal compartments

We have previously shown that the regimen of ChAdOx1-5GHPV3 prime and MVA-5GHPV3 boost administered intramuscularly with an interval of two weeks induces high magnitude vaccine-specific T cell responses, with an immunodominant CD8+ T cell response targeting an epitope (EVYNFAYTDL) within the E6 protein identified [20]. In this study, we employed an H-2K$^b$ pentamer specific to the E6 epitope EVYNFAYTDL to examine how different vaccination routes influence the magnitude, kinetics, and phenotype of vaccine-specific T cells in both systemic and cervicovaginal compartments. Groups of six female C57BL/6 mice were immunised on d0 with 1x10$^8$ IU/ml ChAdOx1-5GHPV3 via intramuscular (IM), intranasal (IN), or intracervical (ICV) administration and boosted on d14 with 1x10$^7$ pfu MVA-5GHPV3 by a heterologous or homologous delivery route (Table 1). Intracervical vaccination was performed using an adapted transcervical embryo transfer (TCET) technique, in which a flexible catheter was used to deposit vaccine into the cervix. Detectable GFP expression by IVIS imaging following ICV administration of ChAdOx1-GFP or MVA-GFP confirmed effective delivery of vaccine to the female genital tract (Supplementary Fig. 1). Splenocytes and cervicovaginal cells were harvested at days 14, 21, and 28 post-prime vaccination and E6-specific T cells were quantified by pentamer-binding (Figure 1A). Prime vaccination with ChAdOx1-5GHPV3, regardless of route, induced only low frequencies of E6-specific CD8+ T cells in the spleen. Among the prime routes, IM administration elicited the highest response (mean [SD] 1.1% [0.07] E6+CD8+ T cells), though this was not significantly greater than that induced by IN or ICV vaccination (mean 0.37% and 0.003%, respectively; Figure 1B).

The route of booster vaccination shaped the kinetic pattern of the E6-specific CD8+ T-cell response in the spleen. Among the three homologous prime-boost regimens (IMIM, ININ, and ICVICV), peak responses were observed on d21 post-prime, with homologous systemic vaccination (IMIM) eliciting a higher magnitude of response, mean [SD] of 5.7% [1.8] E6+CD8+ T cells, compared to both homologous mucosal routes (ININ mean [SD] 3.4% [1.1], ICVICV 0.2% [0.3]). By d28 post-prime these responses had declined to levels comparable to those observed on day 14 post-prime. In contrast, heterologous prime-boost regimens induced a delayed peak, with E6-specific CD8+ T cell responses reaching their highest observed level on d28. Heterologous IMIN elicited the highest percentage of E6+CD8+ T cells (mean [SD] 7.7% [3.2%]) compared to all other vaccination regimens, demonstrating a statistically significant enhancement of the response (Figure 1B). No vaccine-specific CD8+ T cells were detected in mock vaccinated mice (Figure 1B and C).

In the cervicovaginal compartment, prime vaccination by any route induced a higher percentage of E6-specific CD8+ T cells than that observed in the spleen. Following IM ChAdOx1-5GHPV3 prime vaccination, the mean [SD] percentage of vaccine-specific cells in the FRT was 12.9% [3.2], compared with 1.1% [0.07] in the spleen. Mucosal prime vaccination routes (IN and ICV) were less effective at inducing E6+CD8+ T cells in the cervicovaginal compartment than systemic vaccination (mean [SD] 8.5% [3.1] and 3.1% [4.5], respectively). A homologous boost did not further increase the magnitude of the cervicovaginal response elicited by the prime; instead, vaccine-specific CD8+ T-cell frequencies declined between days 14 and 28. For example, following IMIM prime-boost vaccination, 12.9% of CD8+ T cells were E6-specific at day 14 post-prime, declining to 8% by day 28; with similar trends for ININ and ICVICV. By contrast, when

the boost vaccination was delivered by a heterologous delivery route, the magnitude of response increased from d14 to d28, with the highest observed percentage of E6-specific CD8+ T cells observed at day 28.. The highest magnitude of response in the cervicovaginal compartment was achieved following IM prime IN boost, with a remarkable 29% [6.4] of total CD8+ T cells being E6-specific. As observed in the spleen, the IMIN regimen elicited a significantly higher magnitude of response compared than the other heterologous prime-boost regimen, IMICV, ($p = 0.0006$) as well as all homologous prime-boost regimens ($p \leq 0.0001$, Figure 1C).

In summary, intramuscular prime-intranasal boost vaccination elicits the highest magnitude of E6-specific CD8+ T cell responses, in both the spleen and cervicovaginal compartment, compared with other regimens tested.

**Mucosal boosting induces a higher frequency of E6-specific tissue resident CD8+ T cells in the cervix**

To assess how vaccination route influences the differentiation of vaccine-specific CD8+ T cells, we characterised their phenotypes in systemic and cervicovaginal compartments by gating pentamer-specific CD8+ T cells on CD62L and CD127 expression (Supplementary Figure 2). At d14 post IM prime vaccination, E6-specific CD8+ T cells in the spleen predominantly exhibited an effector phenotype ($T_{EFF}$; $CD62L^{-}CD127^{-}$). By d21, irrespective of the vaccination regimen, the frequency of E6-specific CD8+ effector T cells had started to decrease whilst the proportion of effector memory T cells increased ($T_{EM}$ $CD62L^{-}CD127^{+}$), consistent with transition from effector to memory phenotypes during the contraction phase of T cell responses (Figure 2A). At d28 post-

boost, central memory T cells ($T_{CM}$; $CD62L^{+}CD127^{+}$) were observed following vaccination regimens consisting of a systemic prime-mucosal boost, but were not present following homologous systemic prime-boost. In the cervicovaginal compartment, E6-specific CD8+ T cells demonstrated a predominant effector phenotype (Figure 2B). Notably, no vaccination regimen induced a significantly different phenotype profile in either compartment. This uniformity is favourable, as it suggests that while IMIN prime-boost vaccination induced a higher magnitude of vaccine-specific CD8+ T cells, the overall phenotypic composition of the response was comparable across vaccination routes. .

Tissue-resident memory T cells ($T_{RM}$) play a critical role in mediating localised immune responses within the female genital tract. They are defined by the expression of CD69, a marker of tissue retention and the co-expression of CD103, an E-cadherin-binding integrin that further facilitates retention within the tissue microenvironment. We assessed the expression of CD69 and CD103 on pentamer-positive CD8+ T cells to examine the influence of vaccine delivery route on the frequency of $T_{RM}$ in the cervicovaginal compartment. Regimens involving a mucosal delivery route elicited a higher frequency of $T_{RM}$ compared with homologous systemic prime-boost. The highest frequency of E6-specific CD8+ $T_{RM}$ cells was observed following IM prime IN boost, peaking at d28 post-prime vaccination (mean [SD] 9.6% [2.4]). This was a significantly higher frequency of E6-specific $T_{RM}$ cells compared to the frequency induced by homologous systemic, IMIM, and heterologous IMICV regimens, as determined by ordinary one-way ANOVA (IMIN vs IMIM p= <0.0058, vs IMICV <0.0217, Figure 2C).

**Intranasal boosting enhances mucosal homing of total and antigen-specific CD8+ T cells**

An effective therapeutic hrHPV vaccine must induce high-magnitude HPV-specific $CD8^+$ T-cell responses and these T cells must be able to efficiently migrate to and be retained within the genital mucosa. Therefore, we examined how vaccination route influenced expression of the mucosal homing integrin, α4β1, on total and antigen-specific CD8+ T cells.

In naïve mice and at d14 post-prime vaccination, total CD8+ T cells from the spleen exhibited minimal α4β1 integrin expression. By d21 post-prime vaccination, there was significant upregulation of α4β1 integrin on total CD8+ T cells, particularly following the IMIN regimen. By d28 post-prime vaccination, a significantly higher percentage of splenic CD8+ T cells expressed α4β1 following the IMIN regimen compared to all other regimens ($p<0.01$, Figure 3A). In the cervicovaginal compartment, a higher percentage of total CD8+ T cells were positive for α4β1 integrin. As observed in the spleen, at d28 post-prime, the IMIN vaccination induced a significantly higher frequency of total CD8+ T cells positive for this integrin compared to all homologous prime-boost regimens ($p<0.0001$, Figure 3B). Notably, when comparing IMIN to IMIM vaccination, the increased frequency of α4β1 CD8+ T cells in the IMIN group highlights the importance of a mucosal boost in guiding T cell trafficking to the cervicovaginal compartment. This upregulation of α4β1 on total CD8+ T cells suggests that immune activation induced by vaccination can drive the expression of mucosal homing markers on bystander CD8+ T cells.

Analysis of α4β1 expression on antigen-specific CD8+ T cells revealed consistently high levels across all time points and vaccination regimens. In the spleen, over 70% of pentamer-positive CD8+ T cells expressed α4β1, while in the cervicovaginal compartment, this proportion exceeded 80% (Figure 3C and D). This suggests that once antigen-specific CD8+ T cells are generated, they

predominantly express mucosal homing markers regardless of the vaccine regimen or time point. Despite this overall consistency notable differences were observed in the cervicovaginal compartment where IMIN vaccination induced a significantly higher frequency of α4β1+ pentamer-positive CD8+ T cells at d21 post-prime compared to IMIM and IMICV, but not ININ. By d28 post-prime, ININ and IMICV vaccination resulted in a higher percentage of α4β1+ pentamer-positive CD8+ T cells than IMIM, with no significant difference observed between ININ and IMICV. These findings reinforce the importance of a mucosal booster, particularly via the intranasal route, to optimise homing of vaccine-induced CD8+ T cells to the site of disease.

**A systemic prime vaccination is necessary for the generation of polyfunctional vaccine-specific CD8+ T cells**

Beyond their frequency and localisation, the quality of vaccine-induced T cells and their capacity to perform multiple effector functions at the same time is an important determinant of antiviral efficacy. Polyfunctional CD8+ T cells, characterised by their capacity to simultaneously secrete multiple cytokines, are associated with superior viral control in many infection and vaccination models [22–25]. We therefore assessed how different vaccination routes shape the functional profile and polyfunctionality of E6-specific CD8+ and CD4+ T cells.

Splenocytes were stimulated with the E6 peptide EVYNFAYTDL, PHA as a positive control, or medium only as a negative control. Cells were subsequently analysed for the expression of IFNγ, CD107a, TNFα, and IL-2. E6-specific IFNγ production by CD8+ T cells was highest when the prime was delivered systemically, regardless of the route of the subsequent boost, at both d21 and d28 post-prime. At d28 post-boost, IMIN vaccination induced a significantly higher percentage of

IFNγ-producing CD8+ T cells compared to all other vaccination regimens (mean [SD] 6.3% [1.5%] of total CD8+ T cells, p=<0.0001, Figure 4A). This highlights the importance of a systemic prime in eliciting vaccine-specific CD8+ T cells capable of robust cytokine production. A similar trend was observed for E6-specific CD8+ T cells producing TNFα and expressing CD107a, a marker of degranulation (Figures 4B and 4C). Vaccine-specific CD8+ T cells produced low levels of IL-2, with IMIN inducing significantly higher expression than both homologous mucosal prime-boost regimens (Figure 4D).

E6-specific IFNγ+ CD4+ T cells were detected at modest frequencies following all vaccination regimens, peaking at day 21 post-prime and returning to baseline by day 28 (Figure 4E). Interestingly, the proportion of vaccine-specific CD4+ T cells positive for CD107a was highest after the ININ homologous prime–boost regimen (Figure 4F). Frequencies of TNFα and IL-2 positive CD4+ T cells were low across regimens, with no significant differences in the proportion of vaccine-specific cells positive for either cytokine (Figure 4G & 4H).



To assess the impact of different vaccination routes on T cell polyfunctionality, we analysed the simultaneous expression of IFNγ, CD107, TNFα, and IL2 using Simplified Presentation of Incredibly Complex Evaluations (SPICE). IM prime vaccination elicited a higher proportion of polyfunctional CD8+ T cells compared to mucosal prime at d14. Notably, over 25% of E6-specific CD8+ T cells from IM-primed mice co-expressed IFNγ, TNFα, and CD107, indicating enhanced cytotoxic potential. In contrast, mucosal prime vaccination predominantly induced monofunctional CD8+ T cells, with significantly higher frequencies of IFNγ mono-expressing cells observed following IN and ICV prime compared to IM prime (p = 0.03 for both, Figure 4I

(top row), Supplementary Table 1). At day 21 post-prime, mice that received a systemic prime vaccination exhibited significantly higher frequencies of polyfunctional CD8+ T cells co-expressing IFNγ, TNFα, CD107a, and IL-2 than those primed mucosally, irrespective of the boost route ($p<0.05$, Figure 4I (middle row) and Supplementary Table 1). Mucosally primed groups (ICVICV, ININ) displayed limited polyfunctionality and were dominated by monofunctional IFNγ+ CD8+ T cells. By d28, the difference in the frequency of 4-functional IFNγ+TNFα+CD107+IL2+ CD8+ T cells between mucosally and systemically primed mice was no longer statistically significant, however systemically primed groups still demonstrated a broader cytokine response with an increased number of vaccine-specific CD8+ T cells expressing IFNγ+CD107+TNFα+ (Figure 4I). Vaccine-specific CD4+ T cells at all timepoints were predominantly mono-functional expressing either IFNγ or CD107 and there was no significant difference in the functional phenotype between different vaccine delivery regimens (Permutation test not significant, Supplementary figure 3)



## Discussion

An important consideration in vaccine development is the route of delivery, which can influence the magnitude, quality and anatomical distribution of vaccine-specific immune responses. Cell-mediated immune responses in the female genital tract have been associated with the regression of hrHPV-associated lesions, underscoring the importance of inducing T cell responses at the site of disease to maximise the efficacy of a therapeutic vaccine [26–28]. Although systemic vaccination, most commonly intramuscular injection, efficiently elicits circulating T cells, its capacity to generate immune responses at mucosal sites appears limited, as illustrated by several clinical trials

of vaccines against sexually transmitted infections that have shown only modest efficacy despite inducing circulating CD8+ T cells [29,30].

In this study we show that a systemic prime followed by an intranasal boost (IMIN) elicits a favourable immune profile relevant for therapeutic vaccines targeting infections with high-risk HPV types. This regimen generated the highest percentage of E6-specific CD8+ T cells both systemically and in the cervicovaginal mucosa, increased frequencies of tissue-resident memory T cells, enhanced expression of mucosal homing markers, and increased polyfunctionality of CD8+ T cells. In contrast, homologous prime-boost regimens, whether systemic or mucosal, elicited significantly fewer E6-specific CD8+ T cells both systemically and in the cervix, and the CD8+ T cells induced by these regimens produced significantly less CD107a and IFNγ. These findings support a prime-pull model in which systemic antigen-specific CD8+ T cells generated by an IM prime are drawn into and retained at the mucosal site by an intranasal boost [31–34]. Intramuscular priming activates large numbers of naïve T cells within major lymphoid organs and generates a high frequency of circulating effector and memory-precursor T cells, providing a reservoir for subsequent tissue seeding. A subsequent intranasal boost, which engages the nasal-associated lymphoid tissue (NALT) would trigger local antigen presentation and imprint mucosal homing cues, thereby pulling systemic effectors into distant mucosal sites, including the female genital tract, via the common mucosal immune system. Our findings are in line with those of Lorenzen et al., who demonstrated that IM prime-IN boost vaccination of female minipigs induced detectable immune responses in the genital tract and that these were superior to those elicited by homologous IMIM vaccination [35]. Similarly, Çuburu et al. demonstrated that intramuscular priming followed by intravaginal boosting induced strong systemic and tissue-resident CD8+ T

cell responses compared with homologous IVAG/IVAG, IM/IM, or IVAG/IM regimens; however, that study did not evaluate an IM prime followed by intranasal boost [34]. Evidence from human SARS-CoV-2 vaccination studies also supports the importance of mucosal antigen exposure. Robust peripheral immune responses following intramuscular immunisation were frequently accompanied by limited mucosal immunity in the respiratory tract, unless antigen was additionally encountered at the mucosal surface, such as during breakthrough infection. These observations indicate that systemic and mucosal immune responses in humans appear compartmentalised, and that systemic vaccination alone may be inadequate to elicit effective mucosal immunity against pathogens that infect mucosal surfaces [36,37]. In our study, intracervical boosting was less effective than intranasal administration at enhancing genital tract T cell responses, despite evidence of local antigen delivery (GFP imaging). The female reproductive tract presents epithelial barriers to antigen uptake, is characterised by a tightly regulated immunological milieu and lacks constitutive organised mucosa-associated lymphoid tissue. However, recent evidence demonstrates that it can function as an inducible site of cell-mediated immunity through the formation of inducible vaginal lymphoid tissue (IVALT), supporting local CD8+ T cell priming and the establishment of $T_{RM}$ populations [38]. Our findings therefore suggest that, in the context of ChAdOx1/MVA vaccination, antigen presence at the genital mucosal surface may not have been sufficient to initiate IVALT formation and drive robust local T cell expansion and that effective genital tract boosting is highly dependent on vector platform. An additional potential advantage of intranasal compared with intracervical administration of viral vectored vaccines is the reduced risk of inducing inflammation in the female genital tract. Local inflammation in the genital mucosa has been associated with increased susceptibility to HIV acquisition [39,40], a

concern particularly relevant in sub-Saharan Africa where therapeutic HPV vaccines could have substantial public health impact.

We note several limitations. First, this study focused primarily on a single immunodominant E6 epitope (EVYNFAYTDL) in C57BL/6 mice. In outbred populations and human subjects, greater epitope breadth, HLA/MHC diversity, and CD4+ T cell help are expected, which may result in quantitatively different responses. Second, our analyses extend to day 28 post-prime and did not address long-term durability of $T_{RM}$ or memory pools, an essential consideration for therapeutic vaccines intended to effect durable clearance of persistent infections. Third, while intracellular cytokine staining shows polyfunctionality, we did not perform viral clearance / lesion-regression experiments in this study; absence of a suitable hrHPV persistence/lesion model constrained direct efficacy readouts. Fourth, we investigated a ChAdOx1 prime/MVA boost regimen, an extensively studied and well-established heterologous platform known to induce robust T cell responses in humans [19]. We did not investigate the reciprocal strategy of MVA prime/ChAdOx1 boost. The use of ChAdOx1 as a mucosal boost may differentially influence the magnitude, localisation, and quality of T cell responses and warrants direct comparative evaluation in future studies. Despite these limitations, insights from preclinical studies such as ours can help guide the design of next-generation therapeutic vaccine strategies. For example, a recent Phase 1b/2 clinical trial (NCT04607850) evaluated VTP-200, a ChAdOx1- and MVA-vectored HPV therapeutic vaccine, administered intramuscularly in women with persistent hrHPV infection and low-grade cervical lesions. While the highest dose groups showed a trend towards increased hrHPV clearance and lesion regression compared with placebo, these differences did not reach statistical significance (manuscript accepted). Our findings suggest that incorporating an intranasal boost could enhance

immune responses at the mucosal site of disease, potentially improving vaccine efficacy. Evaluation of this approach in future clinical trials is therefore warranted.

Viral vectored vaccines remain a powerful platform for therapeutic vaccination, particularly for inducing robust systemic CD8+ T cell responses, However, emerging data suggest that messenger RNA (mRNA) vaccines may offer additional advantages for therapeutic vaccination against mucosal pathogens such as HPV. mRNA platforms can be rapidly engineered to encode multiple antigens, sustain antigen expression for sufficient duration to support effective T cell priming, and avoid anti vector immunity that may limit repeated boosting with viral vectors. Preclinical studies in other infection models have shown that mRNA vaccination can efficiently prime cytotoxic T cells and when delivered through heterologous routes, enhance recruitment of effector and memory T cells into mucosal tissues. Incorporating mRNA based boosters into prime-pull strategies, either following a viral vector prime or as part of combined mRNA only regimens, may therefore offer a means to further strengthen mucosal CD8+ T cell immunity and improve durability of local tissue resident memory in the female genital tract. Evaluation of such hybrid or mRNA enhanced regimens represents an important future direction for therapeutic hrHPV vaccine development [41–47].

In summary, our data highlights the potential of hybrid vaccination strategies that combine systemic priming with mucosal boosting to overcome limitations of systemic-only approaches and to better target immune responses to tissues relevant for disease control.

**Data Availability**

All data had been included in the manuscript and supplementary material. Source data and used protocols can be requested from the corresponding author.

**Funding Statement**

This research was supported by the John-Fell Fund, University of Oxford.

**Author Contributions**

GH performed the experiments and data analysis. GH prepared the figures. GH and KH jointly wrote the manuscript. TE, LD and CL provided expert input and edited the manuscript. All authors reviewed and approved the manuscript.

**Conflict of Interest**

The authors have no financial or non-financial conflict of interest to declare in relation with this manuscript. KH, GH and CLC are employees of the University of Oxford. LD is an employee of Immunocore and TE is consulting for Barinthus Biotherapeutics. Author KH is a Guest Editor of npj Vaccines Collection 'Therapeutic HPV Vaccines'. They were not involved in the journal's review of, or decisions related to, this manuscript.

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**Tables:**

| | Vaccination Regimen | | Spleen / FGT isolation | | | |
|---|---|---|---|---|---|---|
| | ChAdOx1-5GHPV3 prime (d0) | MVA-5GHPV3 boost (d14) | D0 | D14 | D21 | D28 |
| Group 1 (6 mice) | IM | IM | | x | x | x |
| Group 2 (6 mice) | ICV | ICV | | x | x | x |
| Group 3 (6 mice) | IN | IN | | x | x | x |
| Group 4 (6 mice) | IM | IN | | | x | x |
| Group 5 (6 mice) | IM | ICV | | | x | x |
| Group 6 (6 mice) | Naive | Naive | x | | | |

**Table 1; Vaccination regimens. IM; intramuscular, IN; intranasal, ICV; intracervical**

**Figures:**

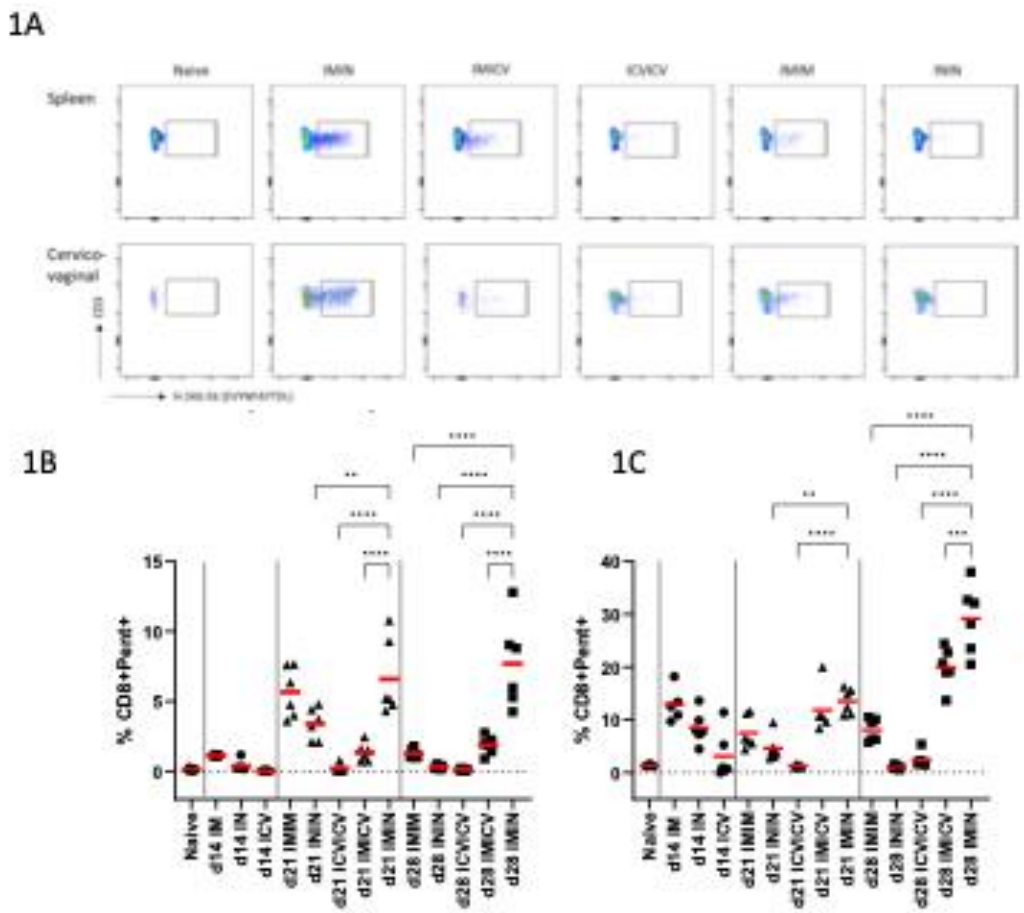
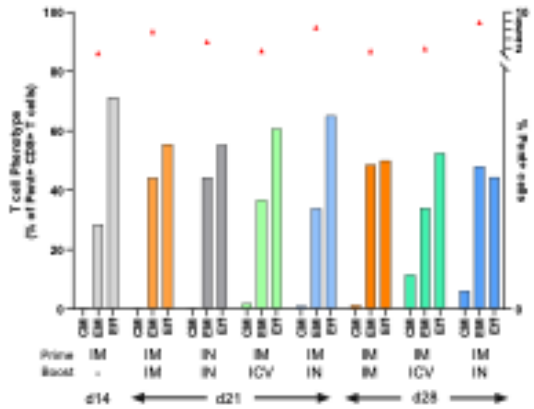



**Figure 1: The route of vaccine delivery impacts the magnitude of the vaccine-specific CD8+ T cell response.** C57BL/6 mice were primed on d0 with $1x10^8$ IU/ml ChAdOx1-5GHPV3 via IM, IN or ICV vaccination and then boosted on d14 with $1x10^7$ pfu MVA-5GHPV3 by a homologous or heterologous vaccination route (IM, IN or ICV). At d0, d14, d21 and d28 cell suspensions from cervicovaginal tissue and spleen were analysed by flow cytometry to quantify H-$2K^b$ $E6_{EVYNFAYTDL}$ $CD8^+$ T cells. (A) Representative plot showing H-$2K^b$ $E6_{EVYNFAYTDL}$ pentamer staining of CD8+ T cells from the spleen (top row) and cervicovaginal compartment (bottom row) at day 28 post-boost. Percentage of CD8+ $E6_{EVYNFAYTDL}$ pentamer+ cells in the spleen (B) and cervicovaginal compartment (C). Horizontal lines show mean. P values (*≤0.05, ** ≤0.01, ***≤0.001, **** ≤0.0001) were determined by one-way ANOVA with post-hoc Tukey analysis.

2A



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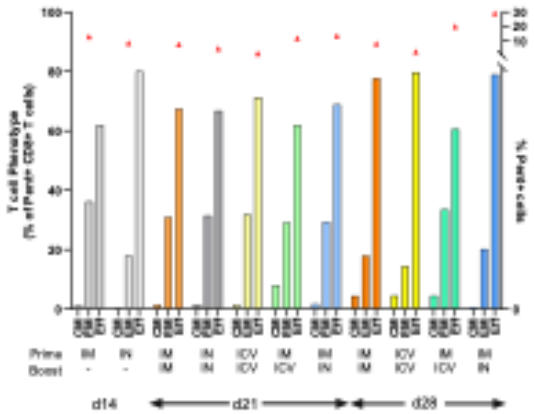

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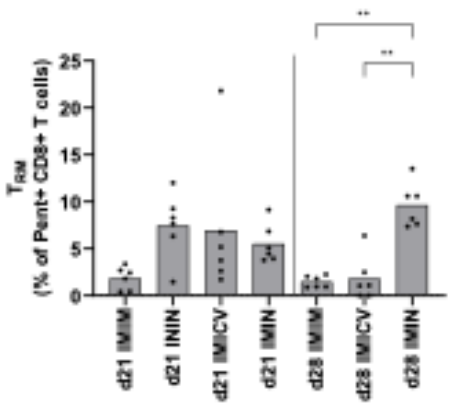


**Figure 2: An intranasal boost induces a higher frequency of E6-specific tissue resident CD8+ T cells in the cervix.** Phenotype of H-2K$^b_{SSEVYNFAYTDL}$ pentamer positive CD8+ T-cells in the spleen (A) and cervicovaginal compartment (B). H-2K$^b_{SSEVYNFAYTDL}$ pentamer positive CD8+ T-cells were gated on CD62L and CD127 expression; $T_{EFF}$; CD62L-CD127-, $T_{EM}$ CD62L-CD127+ and $T_{CM}$; CD62L+CD127+. Red triangles indicate the percentage of H-2K$^b_{EVYNFAYTDL}$ pentamer positive CD8+ T cells. Memory phenotype of pentamer positive cells was determined only for vaccination regimens inducing sufficient numbers of cells for analysis (Mean % of Pent+ CD8+ T cells >1% and SD < mean). Ordinary two-ANOVA with Tukey's multiple comparison test. (C) Percentage of pentamer positive CD8+ Tcells with a $T_{RM}$ phenotype assessed by staining for CD69 and CD103. Bars represent group means overlaid with individual data points. P values (*≤0.05, ** ≤0.01, ***≤0.001, **** ≤0.0001) were determined by one-way ANOVA with post-hoc Tukey analysis.

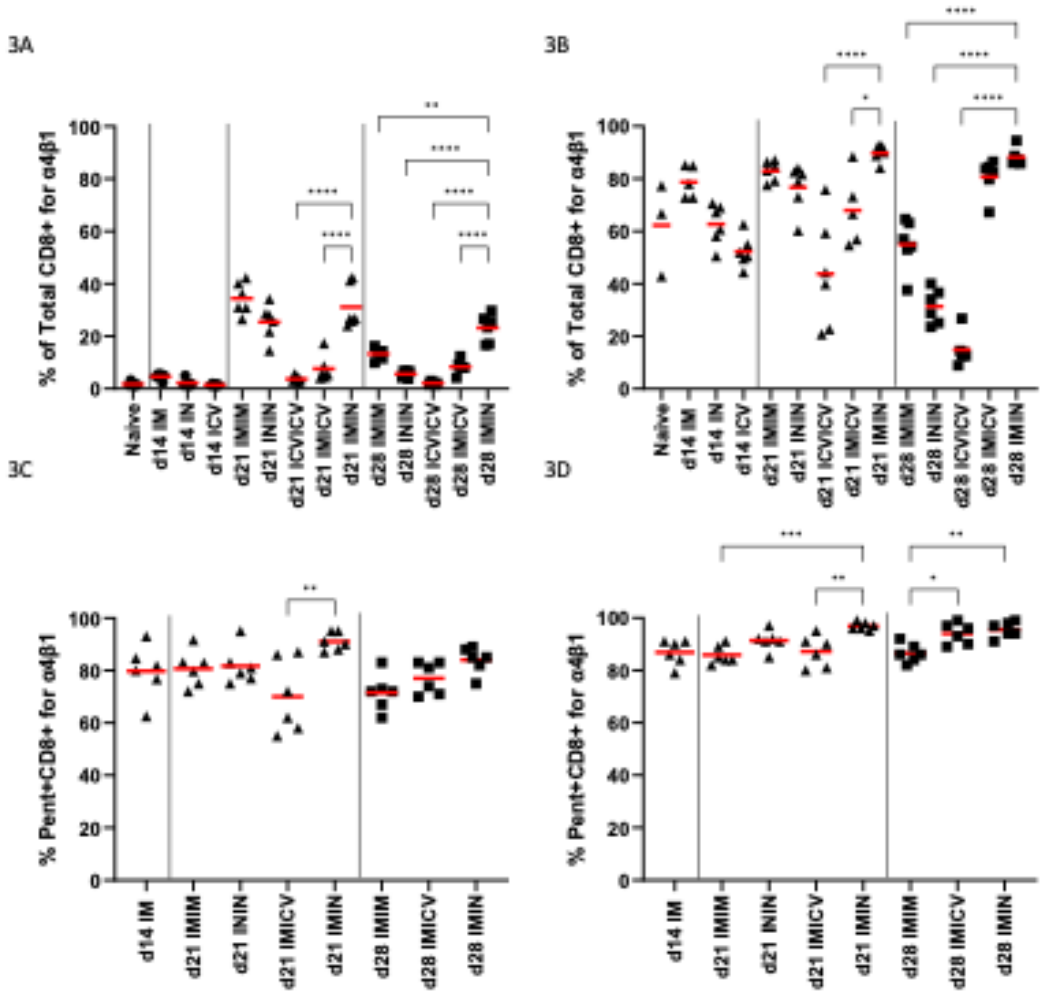


**Figure 3: Systemic prime mucosal boost induces CD8+ T cells with mucosal homing capacity.** α4β1 staining of CD8+ T cells from the Spleen (A) and cervicovaginal compartment (B) and of H-2K$^{b}_{\text{SEVYNFAYTDL}}$ pentamer positive CD8+ T cells from the spleen (C) and cervicovaginal compartment (D). Horizontal lines show mean. P values (*≤0.05, ** ≤0.01, ***≤0.001, **** ≤0.0001) were determined by one-way ANOVA with post-hoc Tukey analysis.

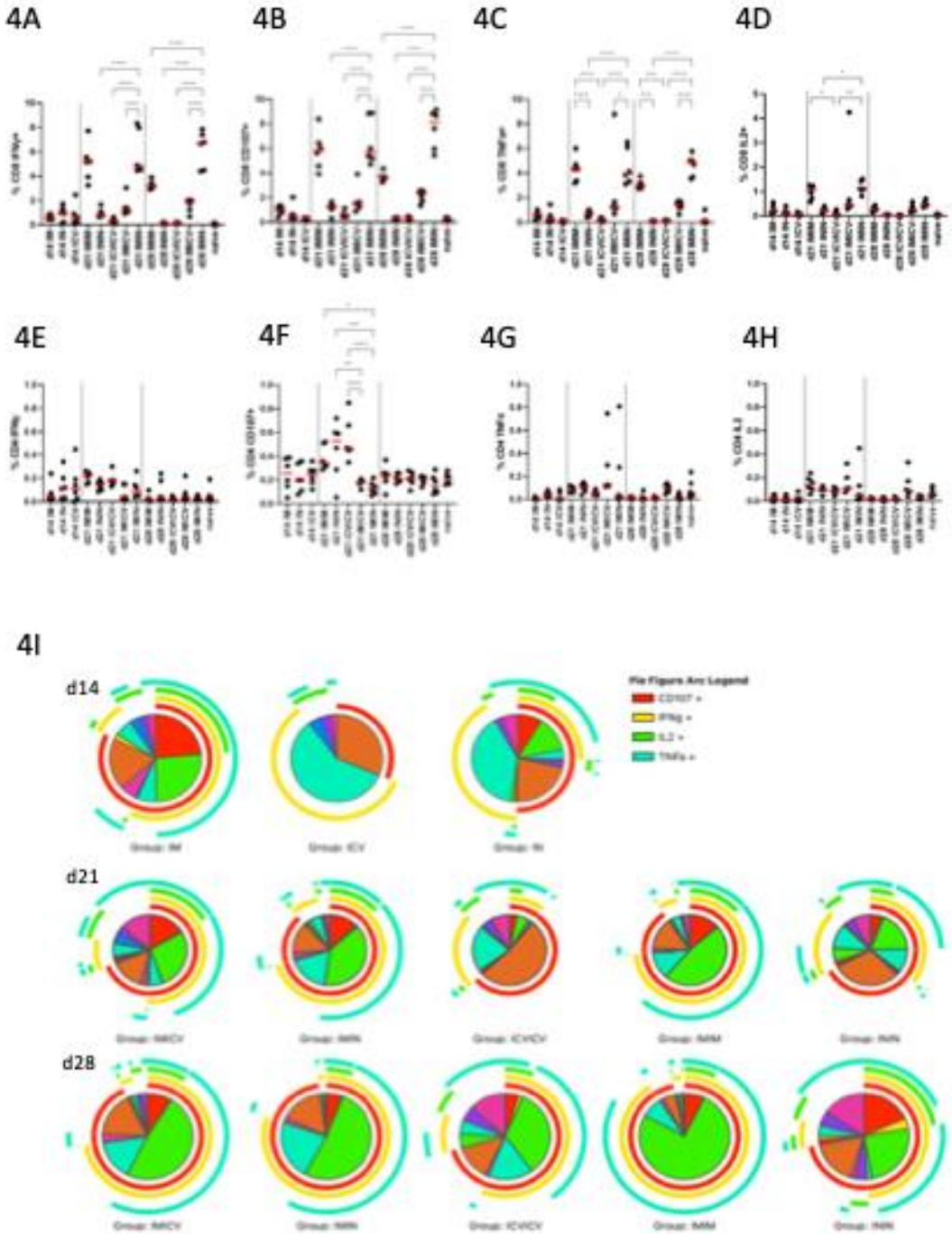


**Figure 4: Systemic priming induces a higher frequency of polyfunctional vaccine-specific CD8+ T cells compared with mucosal priming.** C57BL/6 mice were primed on d0 with $1x10^8$ IU/ml ChAdOx1-5GHPV3 via IM, IN or ICV vaccination and then boosted on d14 with $1x10^7$ pfu MVA-5GHPV3 by a homologous or heterologous vaccination route (IM, IN or ICV). Splenocytes were isolated on days 14, 21, and 28 post-prime and subsequently stimulated in vitro with an E6 peptide (EVYNFAYTDL). Intracellular cytokine staining was performed to measure the production of IFNγ, CD107, TNFα, and IL-2. Percentage of IFNγ (A), CD107 (B), TNFα (C) and IL2 (D) expressing CD8+ T cells after subtraction of background cytokine production of unstimulated CD8+ T cells. Percentage of IFNγ (E), CD107 (F), TNFα (G) and IL2 (H) expressing CD4+ T cells after subtraction of background cytokine production of unstimulated CD4+ T cells. (I) Pie charts show the functional phenotype of E6-specific CD8+ T cells determined using SPICE. Permutation test was performed to assess overall difference in response phenotypes.