Journal of Materials Chemistry B

ROYAL SOCIETY OF CHEMISTRY

PAPER

View Article Online
View Journal | View Issue

Cite this: *J. Mater. Chem. B*, 2024, **12**, 11237

A single-injection vaccine providing protection against two HPV types†

Jianchen Zhang,[a] Yu Liu,[a] Ying Guan *[a] and Yongjun Zhang *[b]

Received 22nd March 2024,
Accepted 25th September 2024

DOI: 10.1039/d4tb00606b

rsc.li/materials-b

Prophylactic human papillomavirus (HPV) vaccines against cervical cancer were successfully developed; however, challenges such as high cost and low compliance still remain to be overcome. In addition, because many HPV types can cause cervical cancer, antigens of multiple HPV types are needed to achieve broad protection. In this study, a bivalent single-injection HPV vaccine was designed in which virus-like particles (VLPs) of HPV 16 L1 and HPV 18 L1 were used as antigens. A recently developed drug carrier that uses tannic acid/polyethylene glycol films as the erodible layer was employed to accomplish multiple pulsatile releases of the antigens. Monovalent single-injection vaccines for HPV 16 and HPV 18 were first designed. A bivalent single-injection vaccine was then obtained by simply mixing the two monovalent vaccines. The bivalent vaccine provided protection against both HPV types. More importantly, it elicited both humoral and cellular immune responses as potent as those elicited by the corresponding multiple dose vaccine because of their similar release profile of antigens. Cross-reactivity was observed between HPV 16 and 18 in terms of cellular immune responses, while no cross-reactivity was found in terms of humoral immune responses. Note that other multivalent single-injection vaccines could be designed in the same way. These vaccines are expected to help prevent cervical cancer because of their broad protection, enhanced compliance and lowered vaccination cost.

Introduction

Human papillomavirus (HPV) is an epithelial cell specific, nonenveloped DNA virus causing many kinds of diseases.[1] In particular, it is associated with cervical cancer, the fourth-most frequent cancer and the leading cause of death by cancer in women. The World Health Organization (WHO) reported that each year 570 000 new cases and 313 000 deaths are caused by this virus.[2] Moreover, about 99.7% of cervical cancer cases have persistent genital HPV infection.[1] Although several prophylactic HPV vaccines have been successfully developed, some challenges still remain to be overcome.[2] One problem is the high price of the vaccines, which prevents their wide application particularly in developing countries.[3,4] The second problem is the multiple dose schedule adopted by current HPV vaccines, which requires the recipient to visit a health-care centre multiple times.[3,5] This schedule has proven to be difficult to follow. It was reported that less than 30% of recipients completed the schedule in the first years of the introduction of the HPV vaccine in the USA.[5] A recent WHO report also revealed a decreased coverage rate of HPV vaccines in 2021 than in 2019, suggesting a low aspiration among people to receive multiple-dose vaccines.[6] Although a systematic review of clinical trial results suggested that one dose of the present commercial HPV vaccine may be as effective in preventing HPV infection as multi-dose schedules in healthy young women,[7] multiple doses are still suggested. Another challenge is that there are more than 200 HPV types.[8] In particular, a number of HPV types, including HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 59, are associated with human cervical cancers.[1] HPV vaccines usually adopt the viral capsid L1 protein virus-like particles (VLPs) as antigens, and their protective effect is limited by the type. Therefore, to achieve extensive protection, several L1 VLPs of different HPV types should be simultaneously used.[2,9,10] For example, the 2-valent HPV vaccine Cervarix uses L1 VLPs of both HPV 16 and 18 to prevent 70% cervical cancers, while the 9-valent HPV vaccine Gardasil 9 for HPV 6/11/16/18/31/33/45/52/58 prevents 90% cervical cancers.[2]

To address problems associated with the traditional multiple-dose vaccines, the concept of single-injection vaccines was proposed.[11,12] These vaccines use novel controlled-release systems to achieve multiple pulsatile releases of antigens over an extended period of time.[11,12] In this way, they not only provide the priming immunization but also the sequential booster immunizations automatically. It is expected that

[a] *Key Laboratory of Functional Polymer Materials, Institute of Polymer Chemistry, College of Chemistry, Nankai University, Tianjin 300071, China. E-mail: yingguan@nankai.edu.cn*

[b] *State Key Laboratory of Separation Membranes and Membrane Processes, School of Pharmaceutical Sciences, Tiangong University, Tianjin 300387, China. E-mail: zhangyj@tiangong.edu.cn*

† Electronic supplementary information (ESI) available. See DOI: https://doi.org/10.1039/d4tb00606b

single-injection vaccines will elicit immune responses comparable to those elicited by multiple doses of the ordinary vaccines although they are administered only once. Compared to the traditional multiple-dose vaccines, the single-injection vaccines will have improved compliance and reduced vaccination costs and may help elevate the vaccination coverage. Unfortunately, owing to unconventional release kinetics, multiple pulsatile release is extremely difficult to achieve.[12] A commonly used strategy is to encapsulate antigens with a biodegradable poly(lactic-*co*-glycolic acid) (PLGA) layer to delay their release.[13–16] However, the fragile antigens tend to be denatured and lose their antigenicity during the encapsulation process.[17] In addition, the complicated degradation behaviours of PLGA[18] make it difficult to achieve distinct pulsatile releases and to accurately control and adjust the lag time. For these reasons, no single-injection vaccine was successfully commercialized after fifty years of efforts.[12]

We recently developed a pulsatile release drug carrier in which dynamic hydrogen-bonded layer-by-layer (LBL) films were used as the erodible coating to achieve delayed release.[19,20] Unlike PLGA, the dynamic LBL films exhibit a very simple erosion behaviour: their erosion rate remains constant throughout the whole process,[21–25] making it facile to achieve distinct pulsatile releases and to control the lag time accurately by film thickness.[20] Single-injection vaccines such as OVA[26] and COVID-19[27] were successfully constructed using the carrier. As expected, the single-injection vaccines elicit similar immune responses to the corresponding multiple-dose vaccines.[26–28] The system was demonstrated to be highly versatile. It allows achieving as many pulsatile releases as possible.[20] When designing the single-injection rabies vaccine to mimic the release profile of the current rabies vaccine, five pulsatile releases instead of three, as in most single-injection vaccines, were achieved.[28] It also allows codelivery of an adjuvant to further enhance the immune responses.[28] Herein, we further demonstrated that it also allows the co-delivery of two antigens, *i.e.*, L1 VLPs of HPV 16 and 18. In this way, a bi-valent single-injection HPV vaccine was successfully designed. Such bi-valent single-injection vaccines requiring only one administration but providing protection against two types of HPV simultaneously may help improve the compliance and hence increase the vaccination coverage. It is worth noting that a single-injection HPV vaccine formulation eliciting a prime-boost immune response was developed recently by Garcea *et al.* using atomic layer deposition.[29,30] LBL assembly was also previously used to design vaccines.[31,32]

Experimental

Materials

The HPV 16/18-L1 VLPs (expressed in *E. coli*) and HPV 16/18 pseudovirus were provided by Biodragon (Suzhou, China). Sodium carbonate (Na_2CO_3, GR) was purchased from Bohai Chemical Industry. Calcium chloride ($CaCl_2$, AR) was purchased from Beijing Chemical Works. Tannic acid (TA) and polyethyleneimine (PEI, M_w = 10 000) were obtained from Aladdin. Bovine serum albumin (BSA) was provided by Solarbio. Aluminium hydroxide and polyethylene glycol (PEG, M_w = 8000) were purchased from Macklin. Cy5-NHS ester was provided by MeiLunBio. Fluorescein isothiocyanate (FITC) was bought from MedChemExpress. Fluorescein-labelled bovine serum albumin (FITC-BSA) was synthesized from FITC and BSA following the manufacturer's instructions. Cyanine5-labelled bovine serum albumin (Cy5-BSA) was synthesized from BSA and Cy5-NHS ester following the manufacturer's instructions. Fluorochrome-labelled anti-mouse antibodies targeting CD3, CD4 and CD8a were obtained from Elabsciense. Roswell Park Memorial Institute (RPMI) 1640 and Dulbecco's modified Eagle medium (DMEM) were supplied by Gibco. The BCA protein assay kit was provided by NJJCBIO. The cell counting kit-8 (CCK-8) was purchased from Biosharp. All mouse cytokine ELISA kits including IL-2, IL-4, TNF-α and IFN-γ were purchased from Meimian. The mouse anti-HPV 16-L1 IgG and mouse anti-HPV 18-L1 IgG ELISA kit were ordered from Alpha Diagnostic. 293FT cells were supplied by Cellverse. Experimental C57BL/6 mice were purchased from Vital River.

Synthesis of the single-injection vaccines

First the antigens were encapsulated in $CaCO_3$ microspheres. For example, to encapsulate HPV 16-L1 VLP in $CaCO_3$ microspheres, 1.0 mg mL^{-1} HPV 16-L1 VLP was prepared using 0.33 M $CaCl_2$ solution. Then, an equal volume of Na_2CO_3 solution (0.33 M) was added and mixed with vigorous stirring (600 rpm) for 2 min. After water rinsing and centrifugation for two times, the resulting HPV 16-L1 VLP-loaded $CaCO_3$ microspheres, HPV 16@$CaCO_3$, were freeze-dried. The same method was used to prepare other antigen-loaded $CaCO_3$ microspheres, including BSA@$CaCO_3$, FITC-BSA@$CaCO_3$, Cy5-BSA@$CaCO_3$ and HPV 18@$CaCO_3$. The protein loading efficiency was determined using a BCA protein assay. The pore size of the $CaCO_3$ microspheres was determined using an ASAP 2460 surface area and porosity analyzer (Micromeritics, USA) by the Brunauer–Emmett–Teller (BET) method.

The antigen-loaded $CaCO_3$ microspheres were then coated with a layer-by-layer TA/PEG film. In brief, a prime PEI layer was first assembled by soaking the antigen-loaded $CaCO_3$ microspheres in PEI solution (1.0 mg mL^{-1}) for 5.0 min, followed by two water rinsing cycles. Then, the TA layer and PEG layer were deposited alternately by soaking the microspheres in TA solution in pH 6.0 phosphate buffer (0.60 mg mL^{-1}) or PEG solution in pH 6.0 phosphate buffer (0.60 mg mL^{-1}), followed by two water rinsing cycles. Finally, the microspheres were freeze-dried. They were labelled as antigen@$CaCO_3$/$(TA/PEG)_n$, where "*n*" refers to the bilayer number of the TA/PEG film. The size and zeta potential of the microspheres were measured by dynamic light scattering (Zetasizer Nano ZS90, Malvern Panalytical).

The monovalent single-injection HPV 16 vaccine, SIV(H16), was then prepared by mixing HPV 16@$CaCO_3$, HPV 16@$CaCO_3$/$(TA/PEG)_{25}$ and HPV 16@$CaCO_3$/$(TA/PEG)_{50}$ (antigen weight 1 : 1 : 1). The monovalent single-injection HPV 18 vaccine, SIV(H18), was prepared by mixing HPV 18@$CaCO_3$, HPV 18@$CaCO_3$/$(TA/PEG)_{25}$ and HPV 18@$CaCO_3$/$(TA/PEG)_{50}$ (antigen weight 1 : 1 : 1). The bivalent single-injection vaccine, SIV(H16 +

H18), was prepared by mixing equal amounts of SIV(H16) and SIV(H18).

Release kinetics

To measure the disintegration kinetics of TA/PEG films, 1.0 mg of $CaCO_3$/$(TA/PEG)_n$ microspheres (n = 5, 10, and 15) were soaked in 5.0 mL of PBS (pH = 7.4) for 5.0 min at 37 °C. After centrifugal separation, the solution was then collected and the equivalent fresh solvent was added. The concentration of TA in the collected solution was measured using a UV-Vis spectrometer (UV-2401PC Shimadzu) at 221 nm. The solution was collected and analyzed at predetermined time intervals until no change was observed in the UV-Vis signal.

To measure the *in vitro* release kinetics of antigens from $CaCO_3$ microspheres, 1.0 mg of FITC-BSA@$CaCO_3$/$(TA/PEG)_n$ microspheres (n = 0, 5, 10, and 15) were soaked in 5.0 mL of PBS (pH = 7.4) at 37 °C. At specific time points, the release solution was completely collected, and an equal volume of pre-warmed fresh solution was added. The quantity of released FITC-BSA was determined using fluorescence spectroscopy (RF-5301PC Shimadzu).

All animal experiments in the study were approved by the Animal Care and Use Committee at Nankai University (2023-SYDWLL-000535). To measure the *in vivo* release kinetics of antigens from $CaCO_3$ microspheres, Cy5-BSA@$CaCO_3$$(TA/PEG)_n$ (dispersed in pH 7.4 PBS) was subcutaneously injected into the hind neck of female C57BL/6 mice (6–8 weeks, n = 12). At predetermined time intervals, the mice were anesthetized using 2% isoflurane and fluorescence images were captured using an IVIS® Spectrum system (Lumina II, Xenogen, USA).

Immune responses

To study the immune responses of the monovalent single-injection HPV 16 vaccine, *i.e.*, SIV(H16), female C57BL/6 mice (6–8 weeks old) were divided into six groups with five mice in each group. The SIV(H16) group received only one shot of SIV(H16) by subcutaneous injection. The PBS, 3 × H16, 3 × (H16 + Al), and 3 × H16@Ca group received three shots of PBS, free HPV 16-L1 VLP, HPV 16-L1 VLP with aluminium hydroxide and HPV 16@$CaCO_3$ at two-week intervals. All vaccines were prepared using pH 7.4 PBS. Except for the PBS group, for all the vaccines, the total dosage of HPV 16-L1 VLP was 30.0 μg. For 3 × H16, 3 × (H16 + Al), and 3 × H16@Ca groups, the dosage of each shot was 10.0 μg.

The blood samples were collected at seven-day intervals. The sera were separated by centrifugation and stored at −20 °C. The level of specific antibodies against HPV 16-L1 was measured with mouse anti-HPV 16-L1 IgG ELISA kit according to the instruction manual.

The level of neutralizing antibodies against HPV 16-L1 was measured by pseudovirus-based neutralization assay (PBNA) using HPV 16 pseudovirus in which a plasmid encoding green fluorescent protein (GFP) was packaged as a reporter gene. Briefly, 293FT cells in logarithmic growth phase were placed in 96-well plates at 15 000 cells each well and cultured with DMEM at 37 °C and 5% CO_2 for 4–6 h in advance. The sera collected 35 and 42 days after the first immunization were serially diluted with four-fold dilution from 10 to 2 621 440 and separately mixed with HPV 16 pseudovirus. After 1 h of incubation at 37 °C and 5% CO_2, the mixture was added into pre-cultured 293FT cells. After an additional 72 h incubation at 37 °C and 5% CO_2, the fluorescent spot of each well was counted by using a fluorescence microscope (IX-70, Olympus). The neutralization of the serum sample was analysed by fitting the curve with Origin. The inhibition rate of HPV 16 pseudovirus was calculated according to the following equation:

$$\text{Inhibition} = (1-(\text{fluorescent spots of diluted sera} - \text{fluorescent spots of blank control})/(\text{fluorescent spots of negative control} - \text{fluorescent spots of blank control})) \times 100\% \quad (1)$$

where the group in which only HPV 16 pseudovirus was added was used as the negative control, while the group in which only the medium was added was used as the blank control.

The mice were sacrificed 42 days after the first injection. The spleens were removed and ground through a 200-mesh cell strainer. The red blood cells were lysed by adding red blood cell lysate and the splenocytes were collected by centrifugation. The splenocytes were cultured with RPMI-1640 medium supplemented with 10% FBS and 1% P/S in 6-well plates and re-stimulated with 5.0 μg mL^{-1} HPV 16-L1 VLP. After 72 h of incubation at 37 °C and 5% CO_2, the cell proliferation was measured with CCK-8 kit. The cytokine levels in the cell supernatant were measured using IL-2, IL-4, TNF-α and IFN-γ ELISA kit. The splenocytes were marked with anti-CD3-APC, anti-CD4-PE and anti-CD8a-FITC, and then analysed by using a flow cytometer (FACS Calibur, BD).

To study the immune responses of the bivalent single-injection vaccine, *i.e.*, SIV(H16 + H18), female C57BL/6 mice (6–8 weeks old) were divided into 10 groups with five mice in each group. The SIV(H16 + H18), SIV(H16) and SIV(H18) groups received only one dose of SIV(H16 + H18), SIV(H16), and SIV(H18), by subcutaneous injection. The PBS, 3 × H16, 3 × H18, 3 × (H16 + H18), 3 × H16@Ca, 3 × H18@Ca, and 3 × (H16@Ca + H18@Ca) groups received three doses of PBS, free HPV 16-L1 VLP, free HPV 18-L1 VLP, a mixture of HPV 16-L1 VLP and HPV 18-L1 VLP, HPV 16@$CaCO_3$, HPV 18@$CaCO_3$, and a mixture of HPV 16@$CaCO_3$ and HPV 18@$CaCO_3$, respectively, at two-week intervals. Except for the PBS group, the total dosage of HPV 16-L1 VLP in the vaccines containing HPV 16-L1 VLP was 15.0 μg, and the total dosage of HPV 18-L1 VLP in the vaccines containing HPV 18-L1 VLP was 15.0 μg. For the groups that received three shots, the dosage of the antigen in each shot was 5.0 μg.

The sera samples were collected in the same way. The levels of specific antibodies against HPV 16-L1 and HPV 18-L1 were measured with mouse anti-HPV 16-L1 IgG ELISA kit and anti-HPV 18-L1 IgG ELISA kit, respectively. The levels of neutralizing antibodies against HPV 16-L1 and HPV 18-L1 were measured by PBNA using HPV 16 pseudovirus or using HPV 18 pseudovirus, respectively.

The splenocytes were also collected and tested using the same procedure as described above, except that 5.0 μg mL^{-1}

HPV 16-L1 VLP and 5.0 μg mL^{-1} HPV 18-L1 VLP were added separately for re-stimulation.

Cytotoxicity

BMDCs were placed in a 96-well plate at 10^5 cells per well. The cells were cultured with RPMI-1640 medium containing different amounts of HPV 16@$CaCO_3$(TA/PEG)$_{50}$ and HPV 18@$CaCO_3$-(TA/PEG)$_{50}$. The weight ratio of two types of microspheres was 1 : 1. After 24 or 48 h of incubation, CCK-8 reagent was added to each well and the absorbance at 450 nm was measured using a Tecan Spark microplate reader. The cell viability was calculated using the following formulation with the normalization method:

$$\text{Cell viability} = (\text{OD value of cocultured with microspheres} - \text{OD value of blank})/(\text{OD value of negative control} - \text{OD value of blank}) \times 100\% \quad (2)$$

Statistical analysis

The experimental data were expressed as mean ± standard error. Statistical analysis was performed using one-way ANOVA (GraphPad Prism) and statistical significance was indicated using * for $p < 0.05$, ** for <0.01 and *** for <0.001.

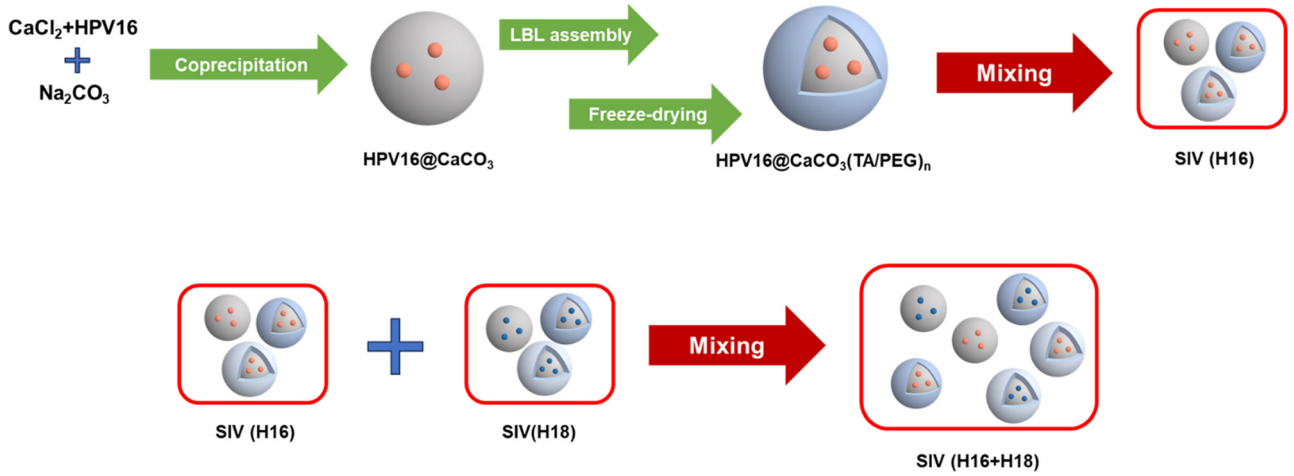
Results and discussion

Release profile of antigens

The bivalent single-injection vaccine was designed using a pulsatile release drug carrier in which TA/PEG LBL films were used as the erodible coating.[19,20] As shown in Scheme 1, first the monovalent single-injection vaccines for HPV 16 and HPV 18, SIV(H16) and SIV(H18), were prepared separately. The bivalent single-injection HPV vaccine, SIV(H16 + H18), was then obtained by simple mixing of the two monovalent vaccines. In both monovalent single-injection vaccines, to achieve their multiple pulsatile release, the antigens were encapsulated in $CaCO_3$ microspheres, which were then coated with different thicknesses of TA/PEG films.[20]

We first used BSA as a model antigen to demonstrate that multiple pulsatile release could be achieved using this system. BSA-loaded $CaCO_3$ microspheres were synthesized by co-precipitation as described by Volodkin *et al.*[33] The average pore size of the $CaCO_3$ microspheres was determined to be 9.9 nm using the BET method. The protein loading efficiency was determined to be about 60% by BCA-kit. The average size and zeta potential of the resulting microspheres were measured by dynamic light scattering to be about 3.58 μm (Fig. S1A, ESI†) and −9.37 ± 0.52 mV (Fig. S1B, ESI†). To coat the microspheres with TA/PEG films, a PEI primer layer was first deposited, leading to the reversal of the surface charge (Fig. S1B, ESI†). After layer-by-layer deposition of TA/PEG films the surface charge was reversed again (Fig. S1B, ESI†). TEM images revealed a core–shell structure of the resulting microspheres (Fig. S2, ESI†). The average thickness of each bilayer was estimated to be ∼10 nm.

Fluorescently labelled BSA FITC-BSA was used to study the *in vitro* release behaviours. We previously demonstrated that, like other dynamic LBL films, the TA/PEG films disintegrate slowly in aqueous solutions because the film materials are bonded with reversible, dynamic hydrogen bonds.[34,35] More importantly, the films disintegrate at a constant rate (Fig. 1A and B), and the disintegration time of the films increases linearly with the film thickness (Fig. S3, ESI†). We previously demonstrated that dynamic LBL films fabricated using polymers with narrow molecular weight distribution disintegrate at a constant rate.[21–25] As shown in Fig. 1C and D, coating the BSA-loaded $CaCO_3$ microspheres with TA/PEG films successfully retarded the release of BSA from the microspheres. The lag time is comparable to the disintegration time of the film, confirming that the release of BSA is controlled by the disintegration of TA/PEG films (Fig. S3, ESI†). The lag time also increases linearly with the film thickness (Fig. S3, ESI†), indicating that it can be facilely controlled by the film thickness.[20] By simple mixing of the microspheres coated with different thickness TA/PEG films, multiple pulsatile releases of BSA were achieved (Fig. 1E and F).[20]



Scheme 1 Synthesis of bivalent single-injection vaccines. The monovalent single-injection vaccines against HPV 16 (SIV(H16)) and HPV 18 (SIV(H18)) were first prepared separately. The two vaccines were then mixed to afford the bivalent single-injection vaccine, SIV(H16 + H18).

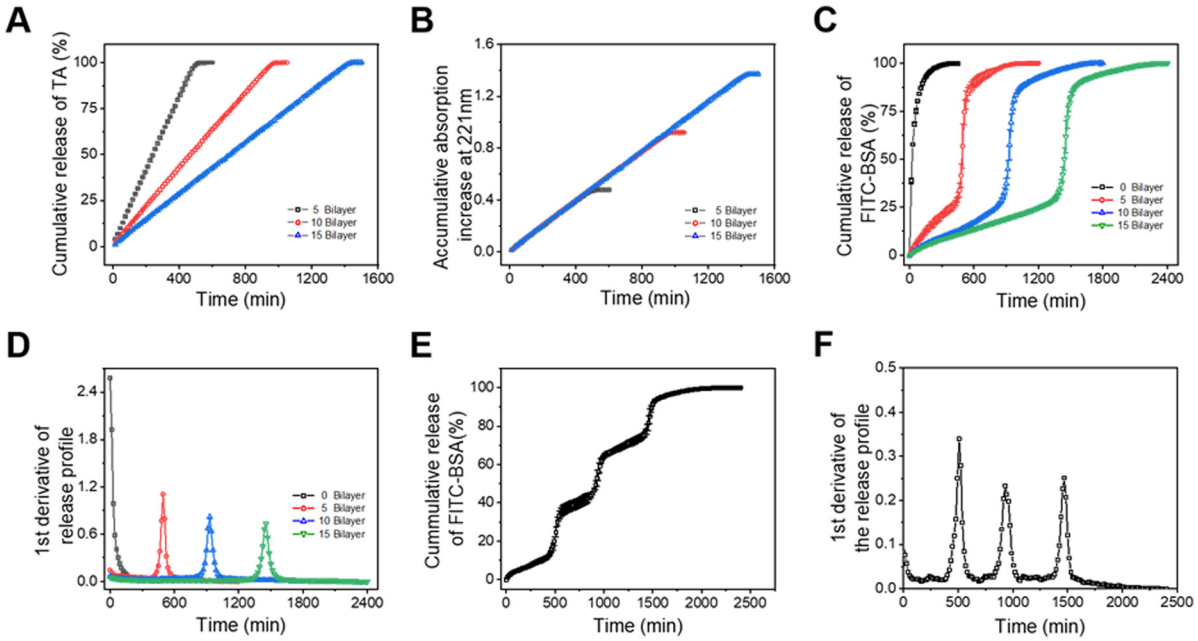


Fig. 1 (A) and (B) Disintegration kinetics of the TA/PEG bilayer films coated on $CaCO_3$ microspheres represented as the cumulative release percentage of TA (A) and cumulative release amount of TA. (C) Release kinetics of FITC-BSA from $CaCO_3$ microspheres coated with 0, 5, 10 and 15 bilayer TA/PEG films. (D) 1st derivative of the release profile in (C). (E) Release kinetics of FITC-BSA from a mixture of $CaCO_3$ microspheres coated with 5, 10 and 15 bilayer TA/PEG films. (F) 1st derivative of the release profile in (E). The medium was 0.01 M pH 7.4 phosphate buffer T = 37 °C.

The *in vivo* release behaviours were studied using fluorescently labelled BSA Cy5-BSA. When the uncoated microspheres were subcutaneously injected into the hindneck of mice, the fluorescent signal was observed immediately, indicating instant release of Cy5-BSA (Fig. 2A) In contrast, a delayed release was successfully observed from the coated microspheres. For example,

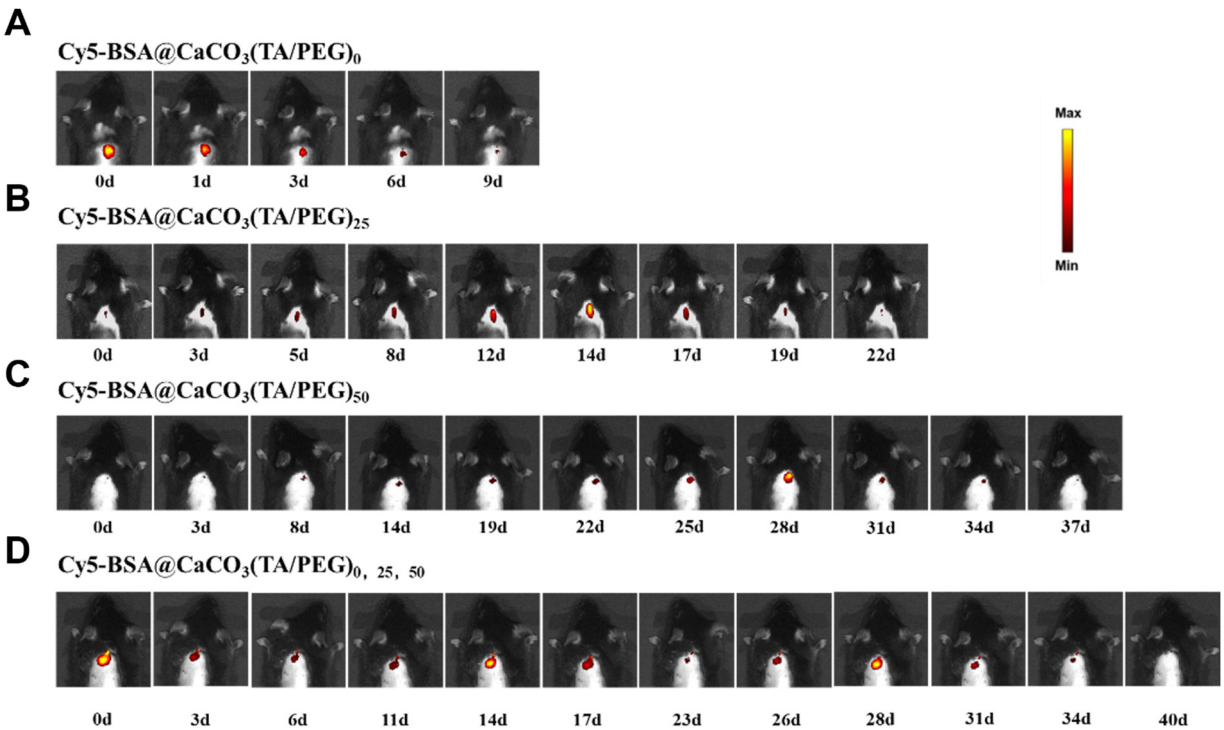


Fig. 2 *In vivo* release of Cy5-BSA from $CaCO_3$ microspheres coated with TA/PEG films. The mice were injected with Cy5-BSA@$CaCO_3$ (A), Cy5-BSA@$CaCO_3$(TA/PEG)$_{25}$ (B), Cy5-BSA@$CaCO_3$(TA/PEG)$_{50}$ (C), and a mixture of Cy5-BSA@$CaCO_3$, Cy5-BSA@$CaCO_3$(TA/PEG)$_{25}$ and Cy5-BSA@$CaCO_3$(TA/PEG)$_{50}$ (D).

the maximum fluorescence signals appeared 14 and 28 days after the injection of the microspheres coated with 25 and 50 bilayer films (Fig. 2B and C). Note that the lag time linearly increases with the bilayer number of the TA/PEG film, providing a good basis for us to adjust the lag time (Fig. S4, ESI†). Moreover, to achieve multiple pulsatile release, one can simply mix the microspheres coated with different thickness TA/PEG films. For example, when mixing Cy5-BSA@$CaCO_3$, Cy5-BSA@$CaCO_3$(TA/PEG)$_{25}$ and Cy5-BSA@$CaCO_3$(TA/PEG)$_{50}$, three pulsatile releases were observed 0, 14 and 28 days after injection (Fig. 2D).

Monovalent single injection HPV 16 vaccine

The monovalent single-injection HPV 16 vaccine, SIV(H16), was designed using HPV 16-L1 VLP as antigens. HPV 16 was chosen because it is the most prevalent type associated with cervical cancers.[10] We also used its viral capsid L1 protein virus-like particle, HPV 16-L1 VLP, as the antigen. First, HPV 16-L1 VLP-loaded $CaCO_3$ microspheres, HPV 16@$CaCO_3$, were prepared by co-precipitation. Microspheres coated with 25 and 50 bilayer TA/PEG films, HPV 16@$CaCO_3$(TA/PEG)$_{25}$ and HPV 16@$CaCO_3$-(TA/PEG)$_{50}$, were then synthesized. The single-injection vaccine was then prepared by mixing HPV 16@$CaCO_3$, HPV 16@$CaCO_3$-(TA/PEG)$_{25}$ and HPV 16@$CaCO_3$(TA/PEG)$_{50}$.

To study its immune responses, the mice in the SIV(H16) group were subcutaneously injected with the single-injection HPV 16 vaccine. For comparison, four other groups of mice were immunized with three doses of PBS, free HPV 16, HPV 16 + Al, and HPV 16@$CaCO_3$, respectively, on days 0, 14 and 28, and were labelled as PBS, 3 × H16, 3 × (H16 + Al), and 3 × H16@Ca groups, respectively. The total dosage of HPV 16-L1 VLP of the HPV 16-L1-containing vaccines was the same.

The sera were collected and HPV 16 L1-specific IgG antibody titers were determined by ELISA. As shown in Fig. 3, except for the PBS group, specific antibodies were detected in all groups immunized with vaccines containing the HPV 16-L1 antigen. The antibody titer was low for the 3 × H16 group, suggesting the relatively weak immunogenicity of the free antigen. Notably increased IgG titers were observed when the second and third doses were administrated on days 14 and 28 (Fig. S5, ESI†). Similar phenomena were previously observed from other HPV L1 VLP-based vaccines.[8] Adding Al as an adjuvant significantly increases the antibody titer by 33% compared to the 3 × H16 group (Fig. 3). In fact, this adjuvant was previously used in HPV vaccines.[8] Interestingly, the encapsulation of the HPV 16-L1 VLP antigen in $CaCO_3$ microspheres significantly increases the antibody titer by about 100% compared to 3 × H16. As revealed in Fig. 2A, encapsulation of the antigen in $CaCO_3$ microspheres leads to sustained release of the antigen. Encapsulation also protects the antigen from enzymatic degradation.[36] Moreover, the microspheres may recruit antigen presenting cells (APCs) and activate dendritic cells (DCs).[28,37] All these explain the significantly enhanced immunogenicity of the antigen encapsulated in $CaCO_3$ microspheres.[28] More importantly, the IgG titer elicited by the single-injection HPV 16 vaccine was equal to that elicited by three doses of HPV 16@$CaCO_3$, although only one dose of the vaccine was administered. According to the results shown in Fig. 2D, the single-injection HPV 16 vaccine will release the antigen in a pulsatile manner on days 0, 14 and 28. The antigen release profile of the SIV(H16) group was actually the same as that of the 3 × H16@Ca group; therefore, similar IgG titers were elicited in both groups.

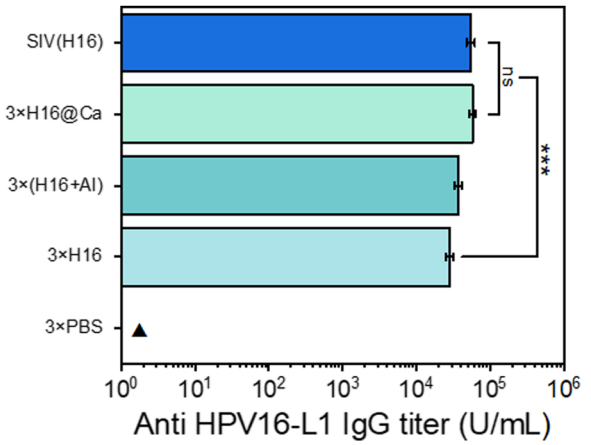


Fig. 3 Anti HPV 16-L1 IgG titer produced in sera 42 days after initial injection. The solid triangle indicates no detectable antibody against HPV 16-L1.

The protective capability of sera from the immunized mice was evaluated by HPV 16 pseudovirus neutralization assays. These assays are considered the gold standard for assessing the protective potential of antibodies induced by the HPV vaccines and included in WHO guidelines.[1,38] As shown in Fig. S6A (ESI†) and Fig. 4A, for sera collected both 35 days and 42 days after initial injection, compared to the 3 × H16 group, 3 × (H16 + Al) group and 3 × H16@Ca group present a higher inhibition rate at the same dilution fold, revealing the adjuvant effects of alum and $CaCO_3$. The single injection vaccine group achieves an inhibition rate close to that of the 3 × H16@Ca group, although three doses were administered in the latter group while only one dose was administered in the former one. Similar trends were observed for neutralization antibody titers (Fig. S6B, ESI† and Fig. 4B). The higher neutralization titers of the 3 × (H16 + Al) group and 3 × H16@Ca group than the 3 × H16 group indicate that co-stimulation with alum or $CaCO_3$ microspheres leads to more efficient protection against HPV 16 pseudovirus. Furthermore, no significant difference in neutralization titers was found for the SIV(H16) group and 3 × H16@Ca group, indicating equal protection provided by the two vaccines.

Interestingly, humoral immune responses HPV vaccines elicit cellular immune responses, which play an important role in fighting against the pathogenic viruses.[8] To assess cellular immune responses, an *ex vivo* splenocyte proliferation assay was performed[36] (Fig. 5A). The immunized mice were sacrificed and the splenocytes were re-stimulated with HPV 16-L1 for 72 h.[39] The higher proliferation index of the 3 × H16@Ca group than the 3 × H16 group suggests that the splenic lymphocytes in this group were activated to a higher level and therefore proliferated more rapidly upon re-stimulation by the same antigen.

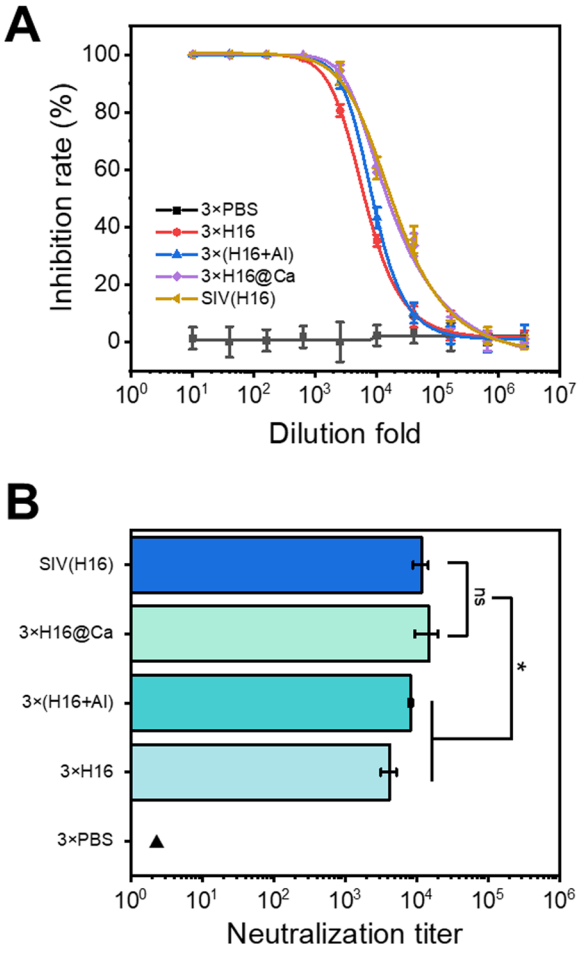


Fig. 4 (A) The inhibition rate of HPV 16 pseudovirus by serially diluted mouse sera. (B) Neutralizing antibody titers against HPV 16 of the sera. The sera were collected 42 days after initial injection. The solid triangle indicates no detectable neutralization reaction with HPV 16 pseudovirus.

The results indicate that the $CaCO_3$ microspheres enhance cellular immune responses. The SIV(H16) group presents a proliferation index close to that of the 3 × H16@Ca group, suggesting a similar level of activation of the splenic lymphocytes in the two groups.

To evaluate the cellular immune responses, cytokine levels secreted by the splenocytes after re-stimulation were measured by ELISA. As shown in Fig. 5B–E, when the alum adjuvant is used or the antigen is encapsulated in $CaCO_3$ microspheres, the secretion of the cytokines, both typical Th1 cytokines (IL-2, IFN-γ and TNF-α[40,41]) and typical Th2 cytokine (IL-4[42]), is up-regulated. The amounts of the cytokines secreted in the SIV(H16) group and the 3 × H16@Ca group are almost equal, indicating that the two vaccines elicited an almost equal level of cellular immune responses.

The lymphocytes were analysed by flow cytometry to evaluate the cellular immune responses elicited by the vaccines. As shown in Fig. S7 (ESI†), a higher percentage of CD4+ and CD8+ T lymphocytes are activated in the 3 × (H16 + Al) group and 3 × H16@Ca group than the 3 × H16 group, confirming again the stronger cellular immune responses when the alum adjuvant and $CaCO_3$ microspheres are used. The almost same population of CD4+ and CD8+ T lymphocytes activated in the SIV(H16) group and the 3 × H16@Ca group confirms that the two vaccines elicit the same level of cellular immune responses.

Bivalent single injection HPV vaccine

After demonstrating that the single injection HPV 16 vaccine, SIV(H16), could induce equal immune responses to conventional three dose vaccines, a bivalent vaccine was further designed. For this purpose, a single injection HPV 18 vaccine using HPV 18 L1 VLP as the antigen, *i.e.*, SIV(H18), was prepared in the same way as the single injection HPV 16 vaccine. Like HPV 16, HPV 18 is prevalent in invasive cervical cancer.[43] The bivalent single injection vaccine (SIV(H16 + H18)) was then obtained by mixing the two monovalent single injection vaccines (Scheme 1). The immune responses of the vaccine were then tested on mice and compared with other vaccines. The 3 × H16, 3 × H18, 3 × (H16 + H18) and PBS groups received three-shots of free antigens (HPV 16 L1 VLP, HPV 18 L1 VLP, and HPV 16 L1 VLP + HPV 18 L1 VLP) or PBS on days 0, 14 and 28. The 3 × H16@Ca, 3 × H18@Ca, and 3 × (H16@Ca + H18@Ca) groups received three-shots of HPV 16@$CaCO_3$, HPV 18@$CaCO_3$, and HPV 16@$CaCO_3$ + HPV 18@$CaCO_3$ on days 0, 14 and 28. The SIV(H16), SIV(H18) and SIV(H16 + H18) groups received only one shot of the corresponding single-injection vaccines.

The blood samples were collected on days 35 and 42. The specific antibodies against HPV 16 L1 or HPV 18 L1 were measured using HPV 16 or HPV 18 IgG ELISA kit. As shown in Fig. S8A (ESI†) and Fig. 6A, no specific IgG antibodies against HPV 16 were detected in 3 × H18, 3 × H18@Ca and SIV(H18) groups. Similarly, as shown in Fig. S8B (ESI†) and Fig. 6B, no specific IgG antibodies against HPV 18 were detected in 3 × H16, 3 × H16@Ca and SIV(H16) groups. As previously reported, there is no cross-immunity between HPV 16 and 18.[44] However, the bivalent vaccines, including 3 × (H16 + H18), 3 × (H16@Ca + H18@Ca) and SIV(H16 + H18), elicit both the specific IgG antibodies against HPV 16 and the specific IgG antibodies against HPV 18 (Fig. S8, ESI† and Fig. 6). In addition, the antibody titer elicited by the bivalent vaccine is comparable to that elicited by the corresponding monovalent vaccine. For example, the anti-HPV 18-L1 IgG titer elicited by 3 × (H16 + H18) is equal to that elicited by 3 × H18 (Fig. S8B, ESI† and Fig. 6B). These results indicate that the body responds to the two antigens independently. As demonstrated above, the HPV 16-L1 antigen encapsulated in $CaCO_3$ microspheres as in 3 × H16@Ca, 3 × (H16@Ca + H18@Ca), SIV(H16), and SIV(H16 + H18) groups elicits a higher anti-HPV 16-L1 IgG titer than the free HPV 16-L1 antigen in the 3 × H16 group (Fig. S8A, ESI† and Fig. 6A). Similarly, 3 × H18@Ca, 3 × (H16@Ca + H18@Ca), SIV(H18), and SIV(H16 + H18) elicit a higher anti-HPV 18-L1 IgG titer than 3 × H18, because the HPV 18-L1 antigen is encapsulated in $CaCO_3$ microspheres (Fig. S8B, ESI† and Fig. 6B). Like the single-injection HPV 16 vaccine SIV(H16), the single-injection HPV 18 vaccine SIV(H18) elicits an anti-HPV 18-L1 IgG titer equal to that of 3 × H18@Ca (Fig. S8B, ESI† and Fig. 6B). More importantly,

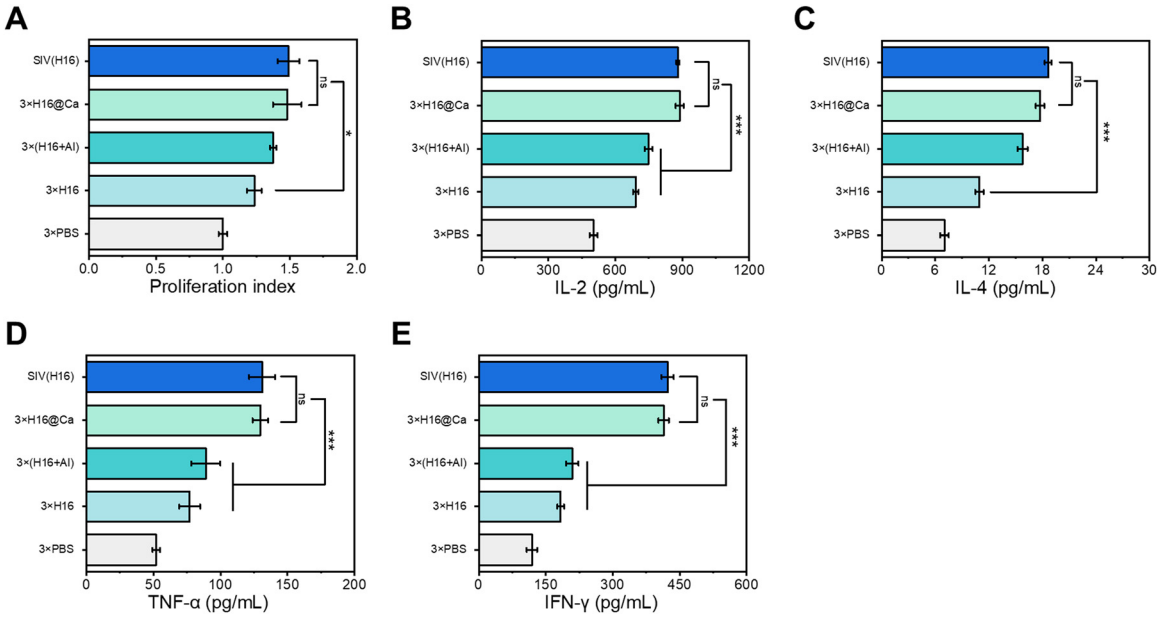


Fig. 5 (A) Proliferation index of splenocytes after 72 h of re-stimulation with HPV 16-L1. (B)–(E) Concentration of cytokines secreted into the supernatant by splenocytes after 72 h of re-stimulation with HPV 16-L1. (B) IL-2, (C) IL-4, (D) TNF-α, and (E) IFN-γ.

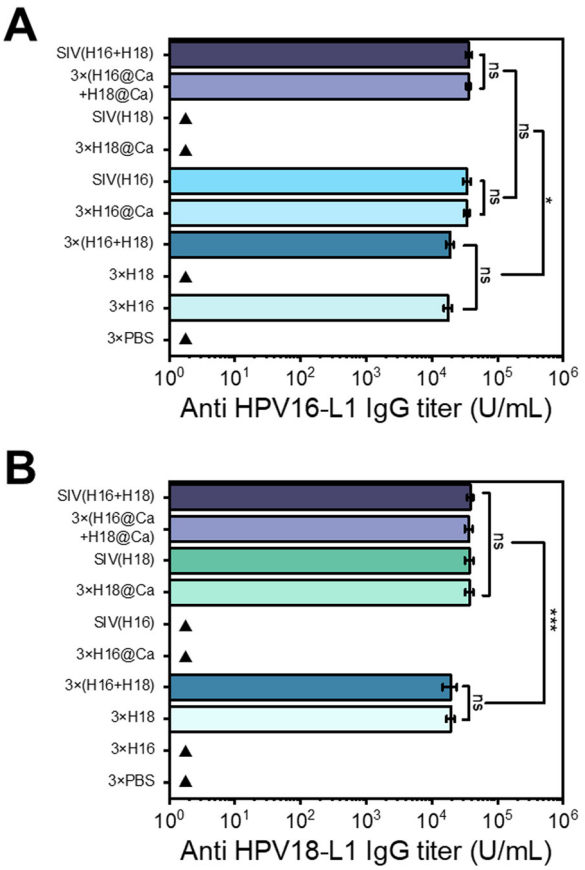


Fig. 6 Anti HPV 16-L1 IgG titer (A) and anti HPV 18-L1 IgG titer (B) produced in sera 42 days after initial injection. The solid triangle indicates no detectable antibody against HPV 16-L1 or HPV 18-L1.

the bivalent single-injection vaccine SIV(H16 + H18) elicits an anti-HPV 16-L1 IgG titer equal to that of SIV(H16) and an anti-HPV 18-L1 IgG titer equal to that of SIV(H18) (Fig. S8, ESI† and Fig. 6).

The protective capability of the immunized sera was then evaluated by pseudovirus neutralization assay.[1,45] In this assay, HPV pseudovirion is incubated with the immunized sera before being added to 293FT cells. The neutralization of the pseudovirion by neutralizing antibodies in the sera will protect the 293FT cells from infection and the non-infected 293FT cells will not express GFP which exhibits green fluorescence. Therefore the level of neutralizing antibodies in the sera can be revealed by the decrease in green fluorescent signal.[1,8] As shown in Fig. S9 (ESI†), treating HPV 16 pseudovirus with sera immunized with vaccines containing HPV 16 L1 VLP (diluted 2560 fold), including 3 × H16, 3 × (H16 + H18), 3 × H16@Ca, SIV(H16), 3 × (H16@Ca + H18@Ca) and SIV(H16 + H18), leads to reduced green fluorescent signals, indicating the generation of neutralizing antibodies against HPV 16 L1. Meanwhile, no significant changes in green fluorescent signals were detected when HPV 16 pseudovirus was treated with sera immunized with vaccines without HPV 16 L1 VLP, *i.e.*, 3 × PBS, 3 × H18, 3 × H18@Ca and SIV(H18), indicating that these vaccines failed to induce neutralizing antibodies against HPV 16 L1. Similarly, the sera were also used to treat HPV 18 pseudovirus (Fig. S10, ESI†). The vaccines containing HPV 18 L1 produce neutralizing antibodies against HPV 18 L1 and therefore protect 293FT cells from infection by HPV 18 pseudovirus, while no neutralization was detected for the vaccines without HPV 18 L1. These results confirm that there is no cross-neutralization between HPV 16 and HPV 18. However, divalent vaccines, *i.e.*, 3 × (H16 + H18), 3 × (H16@Ca + H18@Ca) and SIV(H16 + H18), which provide protection from both HPV 16 pseudovirus and HPV 18 pseudovirus,

could be designed by combination of HPV 16 L1 and HPV 18 L1 antigens.

To determine the neutralizing antibody titers, the sera were diluted serially and the inhibition rate at each dilution factor to both pseudoviruses was measured. For sera immunized with vaccines without HPV 16-L1, *i.e.*, 3 × PBS, 3 × H18, 3 × H18@Ca and SIV(H18), the inhibition rate against HPV 16 pseudovirus approaches zero, indicating no protection against this pseudovirus (Fig. S11A, ESI† and Fig. 7A). In contrast, an almost 100% inhibition rate against HPV 16 pseudovirus was observed for sera immunized with vaccines containing HPV 16-L1, *i.e.*, 3 × H16, 3 × (H16 + H18), 3 × H16@Ca, SIV(H16), 3 × (H16@Ca + H18@Ca) and SIV(H16 + H18), when the dilution factor was lower than 160 or 640 for the sera collected 35 or 42 days post immunization. Similar results were obtained when tested using HPV 18 pseudovirus (Fig. S11C, ESI† and Fig. 7C). The neutralizing antibody titers were calculated and are shown in Fig. S11B, D (ESI†) and Fig. 7B and D. Compared with the vaccines using free antigens, significantly higher neutralizing antibody titers were observed for the vaccines in which the antigen was encapsulated in $CaCO_3$ microspheres (for example 3 × H16 *vs.* 3 × H16@Ca, 3 × (H16@Ca + H18@Ca) and SIV(H16 + H18)). No significant difference was found between the monovalent vaccine and the corresponding bivalent vaccines. For example, the 3 × H16 and 3 × (H16 + H18) groups induced the same neutralizing antibody titers against HPV 16 pseudovirus. These trends are actually similar to the specific antibody response. Previous studies demonstrated a high correlation between specific antibody response and neutralization titer for HPV 16 or HPV 18.[46] Like the bivalent vaccine 3 × (H16@Ca + H18@Ca), the single injection vaccine SIV(H16 + H18) elicits the same high neutralizing antibody titers against HPV 16 pseudovirus and HPV 18 pseudovirus, although only one dose was administrated instead of three doses for the former.

It was thought that neutralizing antibodies are the primary protection mechanism of HPV vaccines;[47,48] however, the level of neutralizing antibodies may decline with time.[10] Therefore potent cellular immune response is highly important for long-term protection. The cellular immune responses of the bivalent single-injection vaccine and the control vaccines were evaluated by *ex vivo* splenocyte proliferation assay (Fig. 8A and F). As shown in Fig. 8F, when the splenocytes were re-stimulated with HPV 18-L1, a rapid proliferation was observed for the groups immunized with vaccines containing HPV 18-L1, indicating that these vaccines generated protective T-cell responses successfully. Again a higher proliferation index was found for the group in which the antigen was encapsulated inside $CaCO_3$ microspheres, for example, the 3 × H18@Ca group, than the corresponding group using free antigens, *i.e.*, 3 × H18, demonstrating again the adjuvant effect of $CaCO_3$ microspheres. A high proliferation index was found for the 3 × H18@Ca

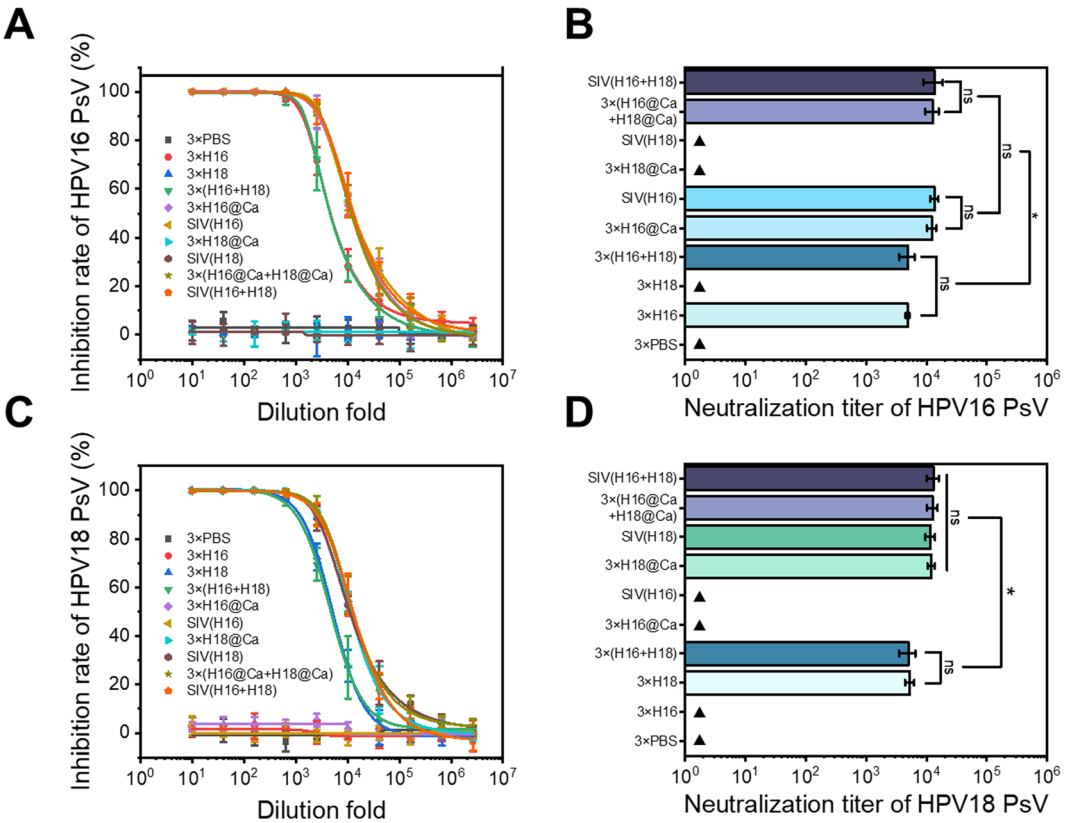


Fig. 7 (A) and (C) The inhibition rate of HPV 16 pseudovirus (A) or HPV 18 pseudovirus (B) by serially diluted mouse sera. (B) and (D) Neutralizing antibody titers against HPV 16 (B) or HPV 18 (D) of the sera. The sera were collected 42 days after initial injection.

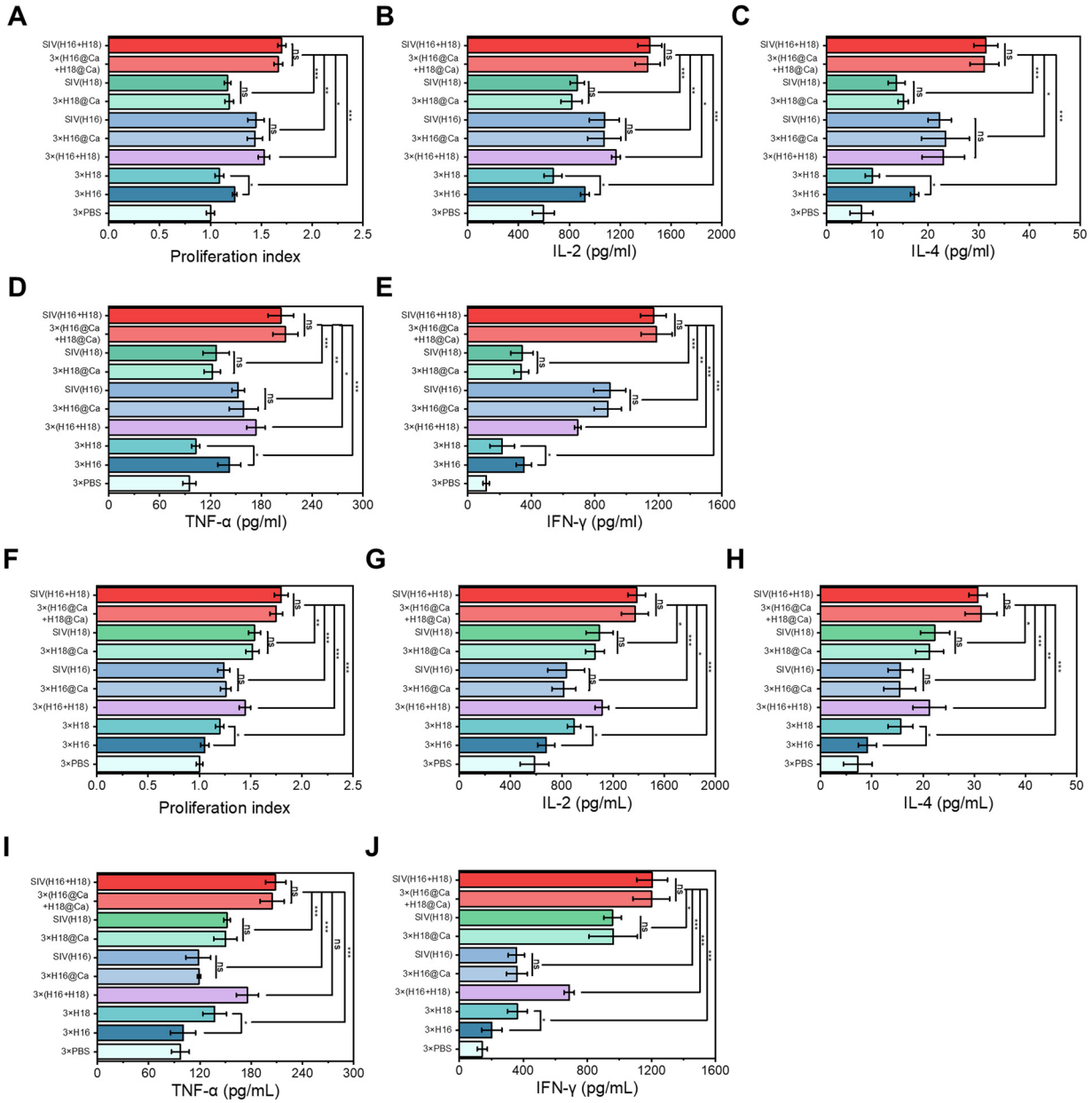


Fig. 8 (A)–(E) Proliferation index (A) of splenocytes after 72 h of re-stimulation with HPV 16-L1, and concentration of cytokines IL-2 (B), IL-4 (C), TNF-α (D), and IFN-γ (E) secreted into the supernatant. (F)–(J) Proliferation index (F) of splenocytes after 72 h of re-stimulation with HPV 18-L1, and concentration of cytokines IL-2 (G), IL-4 (H), TNF-α (I), and IFN-γ (J) secreted into the supernatant.

group and the SIV(H18) group and no significant difference was found between them, revealing that the single-injection vaccine elicited cellular immune response as strong as the three-dose vaccine. In contrast, the groups using HPV 16-L1 as the antigen, *i.e.*, 3 × H16, 3 × H16@Ca, and SIV(H16), exhibited a much lower proliferation index than the corresponding groups using HPV 18-L1 as the antigen, *i.e.*, 3 × H18, 3 × H18@Ca, and SIV(H18), indicating that the cellular response was antigen-specific. It is interesting to observe that, although the proliferation index of the 3 × H16 group is much lower than that of the 3 × H18 group, it was slightly higher than that of the 3 × PBS control group (however there is no statistically significant difference between them). The result may imply a low T-cell cross-reactivity between HPV 16 and HPV 18. A more prominent cross-reactivity was revealed by a significantly higher proliferation index of the 3 × (H16 + H18) group than the 3 × H18 group. The cross-reactivity could also be enhanced by encapsulation of the HPV 16-L1 antigen in $CaCO_3$ microspheres, as revealed by the significantly higher proliferation index of the 3 × H16@Ca group than the 3 × PBS control group. It was pointed out that the number of potential foreign peptide-MHC complexes is much larger than the number of T cell receptors. To provide comprehensive immune coverage each T cell should be able to recognize numerous peptides and therefore

be cross-reactive.[49] Previous studies have demonstrated that a certain HPV vaccine could protect against infection by closely related HPV types but not against more distantly related types.[2,48] For example, the bivalent vaccine Cervarix (against HPV 16 and 18) also provides cross-protection against HPV 31, 33, and 45.[50] As for HPV 16 and HPV 18, previous studies indicated no cross-reactivity between them, because their L1 proteins present a relatively low sequence identity (~66%).[44] However these studies examined only humoral immune responses.[44] Our results shown in Fig. 6 and 7 also confirmed no cross-reactivity between HPV 16 and HPV 18 in terms of humoral immune response. But in terms of cellular immune response, a certain level of cross-reactivity exists between them. It is noteworthy that Helman *et al.*[51] examined if HPV oncoprotein-targeting tumor-infiltrating lymphocytes and T cell receptors possess HPV 16/HPV 18 oncoprotein cross-reactivity and found a low frequency of HPV 16/HPV 18 T cell cross-reactivity. Their results were actually in agreement with the low T cell cross-reactivity between HPV 16 and HPV 18 observed when only free antigens were used.

Similar results were observed when the splenocytes were re-stimulated with HPV 16-L1 (Fig. 8A). In this case, higher proliferation index was observed for the groups using HPV 16-L1 as the antigen, *i.e.*, 3 × H16, 3 × H16@Ca, and SIV(H16), than the corresponding groups using HPV 18-L1 as the antigen, *i.e.*, 3 × H18, 3 × H18@Ca, and SIV(H18), revealing antigen-specific response. Again a low level of cross-reactivity was observed as the proliferation index of the 3 × H18 group was slightly higher than that of the 3 × PBS control group. Enhanced cross-reactivity was observed when the HPV 18-L1 antigen was encapsulated in $CaCO_3$ microspheres (3 × H18@Ca group). The significantly higher proliferation index of the 3 × (H16 + H18) group than the 3 × H16 group also suggested cross-reactivity in the 3 × (H16 + H18) group.

From Fig. 8A and F, when re-stimulated with either HPV 16-L1 or HPV 18-L1, high proliferation index was observed for the 3 × (H16 + H18) group, 3 × (H16@Ca + H18@Ca) group and SIV(H16 + H18) group, indicating that the bivalent vaccines could generate both HPV 16-L1 and HPV 18-L1-specific cellular responses. In addition, there was no significant difference in the proliferation index between the 3 × (H16@Ca + H18@Ca) group and SIV(H16 + H18) group, indicating that the cellular immune response generated by the bivalent single injection vaccine was comparable to that generated by three doses of the conventional bivalent vaccine.

To further investigate T cell immune responses, the cytokine levels in the supernatant, such as IL-2, IL-4, IFN-γ and TNF-α, were determined after re-stimulation (Fig. 8B–E and G–J). When re-stimulated with HPV 16-L1, more cytokines were secreted in 3 × H16, 3 × H16@Ca, and SIV(H16) groups than 3 × H18, 3 × H18@Ca, and SIV(H18) groups (Fig. 8B–E). In contrast, when re-stimulated with HPV 18-L1, more cytokines were secreted in 3 × H18, 3 × H18@Ca, and SIV(H18) groups than 3 × H16, 3 × H16@Ca, and SIV(H16) groups, revealing antigen-specific cellular immune response (Fig. 8G–J). Unlike these monovalent vaccines, high level of cytokines was secreted in 3 × (H16 + H18), 3 × (H16@Ca + H18@Ca) and SIV(H16 + H18) groups when re-stimulated with either HPV 16-L1 or HPV 18-L1, suggesting the successful fabrication of the bivalent vaccines. Compared with the 3 × (H16 + H18) group, 3 × (H16@Ca + H18@Ca) and SIV(H16 + H18) groups secret more cytokines, emphasizing the adjuvant effect of $CaCO_3$ microspheres again. The SIV(H16 + H18) group and the 3 × (H16@Ca + H18@Ca) group secret almost the same level of cytokines, although only one shot was administrated in the former group.

Cross-reactivity was again observed between HPV 16 and 18. Compared with the PBS control, slightly up-regulated cytokine secretions were observed from the 3 × H18 group when re-stimulated with HPV 16-L1 and the 3 × H16 group when re-stimulated with HPV 18-L1. Significantly enhanced cross-reactivity was again observed from the 3 × H18@Ca group when re-stimulated with HPV 16-L1 and the 3 × H16@Ca group when re-stimulated with HPV 18-L1; in both cases, the antigen was encapsulated in $CaCO_3$ microspheres. Prominent cross-reactivity between HPV 16 and HPV 18 was also evidenced by the up-regulated cytokine secretions in the groups of bivalent vaccines compared with the corresponding monovalent groups.

The activation of lymphocytes was further assessed using CD4, CD8 and CD3 as markers. Once again we observed antigen-specific cellular immune response (Fig. 9). When re-stimulated with HPV 16-L1, 3 × H16, 3 × H16@Ca, and SIV(H16) groups present a higher population of CD4+ cells and CD8+ cells than 3 × H18, 3 × H18@Ca, and SIV(H18) groups (Fig. 9A and B). When re-stimulated with HPV 18-L1, however, a higher population of CD4+ cells and CD8+ cells were found for 3 × H18, 3 × H18@Ca, and SIV(H18) groups than 3 × H16, 3 × H16@Ca, and SIV(H16) groups (Fig. 9C and D). In contrast, 3 × (H16 + H18), 3 × (H16@Ca + H18@Ca) and SIV(H16 + H18) groups exhibit a higher population of CD4+ cells and CD8+ cells when re-stimulated with either HPV 16-L1 or HPV 18-L1. Encapsulation of the antigens in $CaCO_3$ microspheres again leads to a higher population of CD4+ cells and CD8+ cells than the corresponding vaccines using free antigens. The SIV(H16 + H18) group exhibits high populations of CD4+ cells and CD8+ cells comparable to that of the 3 × (H16@Ca + H18@Ca) group, confirming that the single-injection bivalent vaccine elicited cellular immune responses comparable to 3 doses of conventional vaccines.

We again observed cross-reactivity between HPV 16 and 18. For example, when re-stimulated with HPV 16-L1 the 3 × H18 group still exhibits a higher population of CD4+ cells than the PBS control (Fig. 9A). An even higher population of CD4+ cells were found for the 3 × H18@Ca group. In addition, the bivalent vaccine groups, *i.e.*, 3 × (H16 + H18), 3 × (H16@Ca + H18@Ca) and SIV(H16 + H18) groups, exhibit a higher population of CD4+ cells than 3 × H16, 3 × H16@Ca and SIV(H16) groups, respectively, which should also be attributed to the cross-reactivity between HPV 16 and 18.

Biocompatibility and stability

To test the safety of the bivalent single-injection vaccine, BMDCs from the femurs and tibias of mice were cultured with

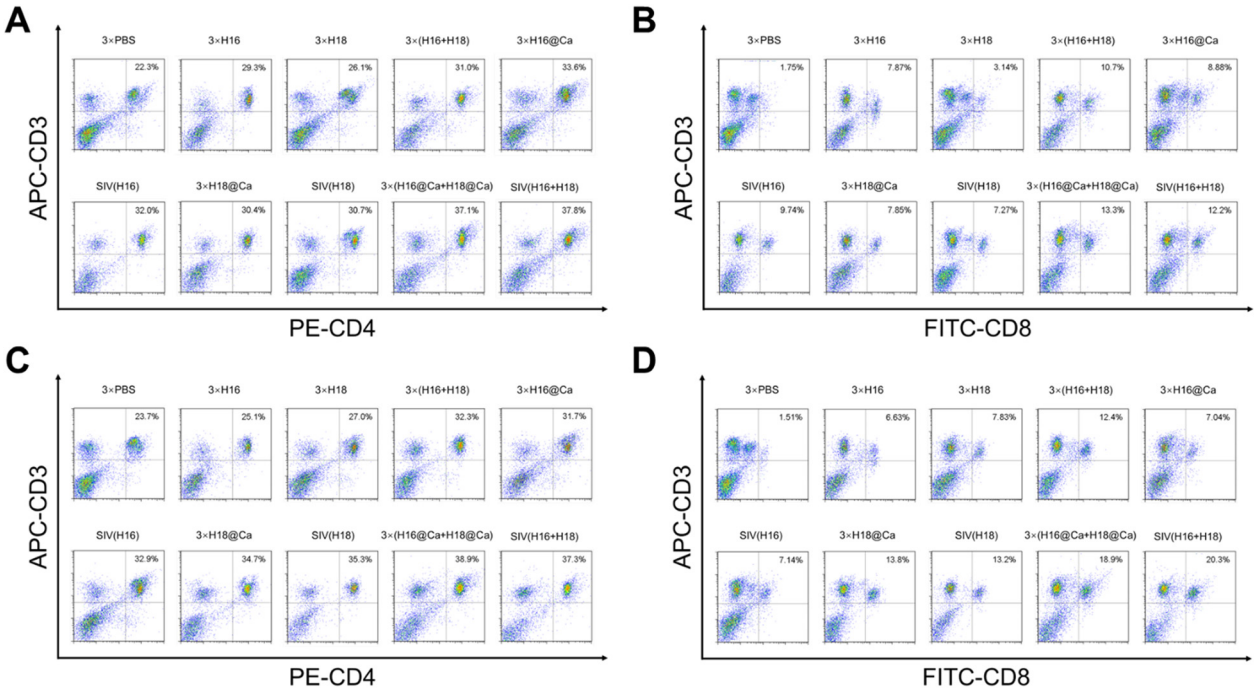


Fig. 9 (A) and (B) Flow cytometric analysis to detect the percentage of CD3+CD4+ (A) and CD3+CD8+ (B) in splenocytes collected from the immunized mice and re-stimulated with HPV 16-L1. (C) and (D) Flow cytometric analysis to detect the percentage of CD3+CD4+ (C) and CD3+CD8+ (D) in splenocytes collected from the immunized mice and re-stimulated with HPV 18-L1.

different doses of the HPV 16@$CaCO_3$(TA/PEG)$_{50}$/HPV 18@$CaCO_3$-(TA/PEG)$_{50}$ mixture in 96-well plates. No significant change in cell viability was found after 24 h and 48 h of coculture, even in the presence of high concentration of microspheres, indicating that the microspheres are non-cytotoxic (Fig. S12A, ESI†). We also followed the body weight change of the immunized mice. As shown in Fig. S12B (ESI†), the body weight of the mice increases with time normally, indicating that immunization with the vaccines does not affect their growth. The immunized mice were sacrificed 6 weeks after the first injection. H&E histological analysis of the main organs including the heart, liver, spleen, lungs and kidneys indicate no obvious damage or inflammation infiltration (Fig. S12C, ESI†). These results indicate that the vaccines developed here are safe. In fact, the vaccine components, including $CaCO_3$, TA and PEG, are all non-toxic and highly biocompatible.

To study the stability of the single-injection vaccine SIV(H16 + H18), it was stored under ambient conditions for 1 month. As shown in Fig. S13 and S14 (ESI†), the sample after 1 month of storage elicits immune responses in terms of both specific IgG antibody titers determined by ELISA and neutralizing antibody titers measured by pseudovirus neutralization assay. The high stability of the vaccine may be attributed to the fact that the antigens are encapsulated in $CaCO_3$ microspheres and stored in solid form.

Conclusions

In conclusion, a bivalent single-injection HPV vaccine was successfully designed using HPV 16 L1 VLP and HPV 18 L1 VLP as antigens and a novel drug carrier which uses TA/PEG films as the erodible layer to control the release of the antigens. The constant erosion rate of TA/PEG films makes it easy to achieve multiple pulsatile releases and accurate control over lag time. First monovalent single-injection vaccines using only one antigen were designed. The single-injection vaccines elicited both humoral and cellular immune responses as potent as three doses of conventional vaccines. The bivalent single-injection vaccine was then obtained by simply mixing the monovalent vaccines. As expected, it provided protection against both HPV types. The humoral and cellular immune responses elicited by this vaccine were comparable to that elicited by the corresponding multiple dose vaccine. Other multivalent vaccines could be designed using the same method. It is expected that these multivalent vaccines will help elevate vaccination rate because of their enhanced compliance and lowered vaccination cost.

Author contributions

Conceptualization: Yongjun Zhang and Ying Guan. Investigation: Jianchen Zhang. Writing – original draft: Jianchen Zhang and Yu Liu. Writing – review and editing: Yongjun Zhang and Ying Guan.

Data availability

The data supporting this article have been included as part of the ESI.†

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

We thank the National Natural Science Foundation of China for financial support for this work (Grants Nos: 52033004, 52073146, 52273109 and 51873091).

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