Thermostability of a Trivalent, Capsomere-Based Vaccine for Human Papillomavirus Infection

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Abstract

Currently licensed vaccines require a cold-chain to maintain efficacy. This cold-chain requirement reduces the availability of vaccines in resource-poor areas of the world. Commercially available human papillomavirus (HPV) vaccines protect against the most common HPV types related to cervical cancer; however, their impact is limited in many regions due to cold-chain requirements. The goal of this study was to test the thermostability of an adjuvanted, trivalent HPV L1 capsomere-based vaccine (containing HPV types 16, 18, and 31) that was formulated by using lyophilization to embed the antigens within a solid, glassy matrix. Thermal stabilities were determined by storing the vaccine formulations for 3 months at 50 °C, followed by immunization of BALB/c mice and measurement of antibody responses. Antibody responses to capsomere vaccines formulated with alum were unchanged after storage for 3 months at 50 °C. Neutralizing responses to these vaccines were unchanged by high-temperature storage, and were equivalent to those generated after administration of the commercially available liquid HPV vaccine Gardasil®9.

Introduction

Many commercially-available vaccines are formulated as liquids that require continuous refrigeration at 2-8 °C (*i.e.*, the "cold-chain") to maintain their stability throughout transportation and storage[1-3]. In developing countries, this cold-chain is not always reliable, and is estimated to contribute up to 20% of the cost of immunization programs [2, 4, 5]. Thermostable vaccines have the potential to both reduce cost of immunization and significantly broaden the availability of protection against preventable diseases[5, 6].

Virtually all cases of cervical cancer are attributable to infection by one of 15 high-risk types of human papilloma virus (HPV)[7]. Infection by HPV is also linked to anal, vaginal, vulvar, penile, oropharyngeal, and skin cancers[7-9]. Highly effective vaccines directed against infection by the most prevalent oncogenic HPV types are now commercially available, but most cervical cancer deaths continue to occur in developing countries[10, 11], where the lack of reliable cold-chain infrastructure and the high cost of the vaccines limit their widespread use[12-14]. An inexpensive, thermostable vaccine alternative would have a major impact in reducing the incidence of cervical cancer in these regions[14].

Commercially available HPV vaccines are based on virus-like particles (VLPs) comprised of 72 L1 pentameric capsomeres, which are purified after expression in yeast of the type-specific L1 protein[15]. VLP-based HPV vaccine formulations are very immunogenic, but the yeast-expressed L1 VLPs require initial purification and subsequent *in vitro* disassembly and reassembly processes to prepare the final VLP antigen[15, 16]. L1 capsomeres can be purified at high yield after L1 expression in *E. coli*[17], do not require the complicated disassembly and reassembly steps and offer immune responses equivalent to VLPs when formulated with adjuvants such as monophosphoryl lipid A (MPLA).[18]

Adjuvants provide an additional challenge for the design of thermostable formulations because the stability of both the antigen and the adjuvant must be maintained. For example, freezing of vaccine formulations adjuvanted with microparticulate aluminum hydroxide (often referred to as "alum"[19]) may cause aggregation of the adjuvant microparticles and result in a loss of vaccine efficacy[20-23]. Loss of vaccine efficacy might also occur after high-temperature excursions from

the cold-chain conditions, which would be expected to accelerate protein degradation and destabilize lipopolysaccharide-based adjuvants, which are often formulated as emulsions[24] .

One strategy for creating thermostable vaccine formulations is to lyophilize vaccine components in mixtures that contain disaccharides. During the lyophilization process, these disaccharides may form glassy matrices that envelop and protect embedded antigens and adjuvants from both physical and chemical degradation[25, 26]. Molecular motions within these glassy matrices are greatly slowed compared to the liquid state, resulting in increased antigen stability[27, 28]. Furthermore, if high concentrations of glass-forming excipients are used in conjunction with fast cooling rates, undesirable phase changes and freezing-induced damage to adjuvants may be minimized during the lyophilization process[25]. In earlier work, we demonstrated that a number of adjuvanted vaccines could be stabilized by lyophilization, including vaccines against ricin toxin, botulinum toxin, Ebola glycoprotein and anthrax[26, 29, 30,31]. Most recently, we developed a lyophilized, adjuvanted vaccine formulation of HPV16 L1 capsomeres that showed complete retention of immunogenicity even after three months of storage at 50 °C[32].

The thermostable, adjuvanted, single–type HPV16 LI capsomere formulation described by Hassett *et al*. [32] would be expected to protect against infection of HPV type 16, the oncogenic HPV type responsible for ≈ 50% of current cervical cancers[33, 34]. A trivalent HPV vaccine including L1 capsomeres from the three most prevalent oncogenic strains (HPV types 16, 18, and 31) would broaden protective immunity to prevent > 70% of cervical cancer cases[35]. Our primary goals in the current work were to 1) determine the thermostability of a lyophilized, adjuvanted, multivalent HPV vaccine containing LI capsomeres from HPV types 16, 18 and 31, and 2) determine whether mixing of these antigens interferes with individual immune responses to each type.

We first tested whether adjuvanted, monovalent formulations of HPV L1 capsomeres from HPV types 18 and 31 could be thermostabilized using lyophilization processes similar to those previously shown to stabilize HPV16 L1 capsomeres[32]. To determine thermostability, antibody and neutralizing antibody

responses in mice to vaccine formulations administered after storage for 3 months at 50 °C were compared to responses generated by material that had not been exposed to high temperatures. Then, using similar measures of thermostability, we addressed the challenge[35] of developing a thermostable, adjuvanted, multivalent HPV vaccine formulation that combined L1 capsomeres from types HPV 16, 18, and 31.

Materials and methods

Materials. High purity, low endotoxin α, α-trehalose dehydrate was a generous donation from Pfanstiehl Inc. (Waukegan, IL). L-Histidine monohydrochloride monohydrate USP grade was from Research Products International Corp. (Prospect, IL). Triethanolamine and bovine serum albumin (BSA) were purchased from Sigma-Aldrich (St. Louis, MO). Two percent aluminum hydroxide adjuvant (Alhydrogel®) was from Brenntag Biosector (Frederikssun, Denmark). Gardasil®9 vaccine was obtained from the University of Colorado, Boulder apothecary. Synthetic monophosphoryl lipid A (MPLA) adjuvant was obtained from Avanti Polar Lipids Inc. (Alabaster, AL). HyClone™ Water for Injection (WFI) was purchased from GE Healthcare (Chicago, IL). Glass lyophilization vials (3 mL), chlorobutyl rubber stoppers, and aluminum caps were purchased from West Pharmaceutical Services (Lititz, PA). 10X concentrated phosphate buffered saline (PBS), Tween80 and sulfuric acid were from Fischer Scientific (Fair Lawn, NJ). 3, 3', 5, 5'-tetramethylbenzidine (Ultra TMB) was purchased from KPL (Milford, MA). Horseradish peroxidase-conjugated donkey anti-mouse antibody was obtained from Promega (Madison, WI).

Expression and purification of recombinant HPV16, 18, and 31 L1 capsomeres. HPV18 and 31 L1 capsomere protein expression was induced by IPTG in HMS174 *E. coli* cultured in shake flasks following protocols reported previously for the expression of HPV16 L1[18] [19]. Bacterial cells were resuspended in 200 mM NaCl Tris buffer pH 8.1 and lysed by two passages through a GEA Niro Soavi Panda homogenizer

(Bedford, NH) operated at 800–1000 bar. An initial chromatographic purification of the soluble fraction of the lysate was conducted on a Q Fast Flow (QFF) column (GE Healthcare, Piscataway, NJ). L1 protein in the flow-through from the QFF column was precipitated with 30% ammonium sulfate, bound on a Q High Performance (QHP) column (GE Healthcare, Piscataway, NJ), then eluted using a sodium chloride gradient. Prior to formulation, L1 capsomeres were exchanged into 100 mM histidine buffer pH 7.1 using size exclusion chromatography.

HPV L1 vaccines without adjuvants. Vaccines were formulated in 54 mM histidine pH 7.1 with 9.5 wt. % trehalose. Monovalent vaccine formulations contained 0.1 mg/mL HPV16, 18 or 31 L1 capsomeres, and trivalent formulations contained 0.05 mg/mL each of HPV16, 18 and 31 L1 capsomeres.

Alum-adjuvanted HPV L1 vaccines. Alum-adjuvanted vaccines were of the same composition as those described above, but with addition of 0.5 mg/mL Al from aluminum hydroxide. These formulations were mixed in 1.7 mL polypropylene tubes (Thermo Fisher Scientific, Hampton, NH) and slowly rotated end-over-end for 1 hour at 4 °C to allow adsorption of the capsomeres to the aluminum hydroxide.

HPV L1 vaccines adjuvanted with alum and MPLA. Freeze-dried MPLA was reconstituted with an aqueous solution of 0.2% (by volume) triethanolamine to form a suspension containing 1 mg/mL MPLA. The suspension was swirled in a 65°C water bath for 1 minute and sonicated in a 25°C water bath for 1 minute. The MPLA-aluminum hydroxide suspension and vaccine formulations described above were mixed together by end-over-end rotation for 1 hour at 4°C to create vaccine formulations containing 0.1 mg/mL HPV L1 capsomere (type 18 or 31), 0.5 mg/mL Al from aluminum hydroxide, and 0.05 mg/mL MPLA in 54 mM histidine pH 7.1 with 9.5 wt.% trehalose. Trivalent vaccines adjuvanted with MPLA and alum were formulated the same way as the monovalent vaccines, but contained 0.05 mg/mL each of HPV16, 18, and 31 L1 capsomeres, 1 mg/mL Al from Alhydrogel®, and 0.1 mg/mL MPLA.

Vaccine lyophilization. Lyophilization of vaccine formulations was performed as described previously[25, 32]. Briefly, 1 mL aliquots of formulations were added to 3 mL lyophilization vials, which were then placed on shelves of a Lyostar freeze-dryer (FTS Systems Lyophilizer, Warminster, PA) that had been pre-cooled to -40°C and allowed to freeze. To initiate primary drying, the shelf temperatures were raised to -20°C and chamber pressure was reduced to 60 mTorr. Temperature and pressure were held constant for 20 hours to allow sublimation of ice. After primary drying, the shelf temperature was increased to 0°C at a rate of 0.2°C/minute, and then to 30°C at a rate of 0.5°C/minute. The temperature was held 30°C for 5 hours to complete secondary drying. Vials were backfilled with nitrogen, stoppered under nitrogen in the lyophilizer using rubber stoppers, and sealed with aluminum crimps. Lyophilized vaccine samples that were not used to evaluate thermostability were stored at -80°C until use.

Water content analysis. Following lyophilization, residual moisture contents in vaccine formulations were determined by volumetric Karl Fischer titration (784 KFP Titrino, Metrohm, Herisau, Switzerland). Hydranal™-Composite 1 (Honeywell Fluka, Morris Plains, NJ) was used as the titrant and Hydranal™-Methanol Dry (Honeywell Fluka, Morris Plains, NJ) was used as the solvent. A solution of 50% (by volume) of methanol and formamide was used to reconstitute lyophilized formulations. Prior to use, residual water levels in the reconstituting solvent were measured; these values were subtracted from sample measurements. Residual moisture was determined in triplicate in a 701 Ti Stand with stirrer and pump (Metrohm, Herisau, Switzerland). After lyophilization, the residual water content in the vials was 0.26 ± 0.05 wt. %.

Incubation of vaccine formulations at elevated temperatures. After lyophilization, samples of each formulation were stored at 50 °C for 3 months. A control formulation, the commercially-available Gardasil®9 HPV vaccine, was also stored at 50 °C for 3 months.

Determination of immune responses in mice. Murine immunization studies were performed at the University of Colorado at Boulder under the Institutional Animal Care and Use Committee protocol #2318. Female BALB/c mice of ages 6-7 weeks were received from Taconic Farms (Hudson, NY). Mice were allowed to acclimate in the vivarium for at least one week before beginning the study. At the start of blood sample collection and immunizations (day 1), mice were 7-8 weeks old. Blood samples were collected by submandibular bleeds with 5.5 mm Goldenrod lancets (Medipoint Inc., Mineola, NY) on days 1, 22, and 37. Approximately 200 μL of blood was collected for each bleed into sterilized 1.7 mL polypropylene tubes. Serum was separated by centrifuging the tubes at 2000g for 10 minutes at 4 °C, and then stored in an -80 °C freezer.

On days 1 and 22, after blood samples were collected, mice were vaccinated intramuscularly under isoflurane anesthesia. Mice receiving reconstituted monovalent HPV L1 capsomere-based vaccines and Gardasil®9 were given 50 μL injections in their left thigh for the first injection and their right thigh for the second injection. Each 50 μL dose of Gardasil®9 contained 6, 4 and 2 μg of HPV16, 18, and 31 VLPs, respectively. Trivalent HPV L1 capsomere-based vaccines were administered by injecting 50 μL in each thigh. Vaccines that had been lyophilized were reconstituted with water for injection to their original volume. Soluble components of the lyophilized vaccines were observed to dissolve rapidly on reconstitution. Prior to injection, all vaccines were swirled gently to ensure suspension homogeneity.

Enzyme linked immunosorbent assay (ELISA). ELISAs to determine serum levels of antibodies against the various capsomeres were carried out using previously established protocols[18]. Briefly, the antigen of interest was plated in 96-well plates at a concentration of 0.5 μg/well in a volume of 150 μL/well and incubated overnight at 4 °C. The next day, plates were washed 3 times with wash buffer (0.1% Tween20 in 1XPBS) then blocked with 1% BSA in wash buffer. Sera were diluted in blocking buffer prior to adding to Nunc MaxiSorb plates. Sera collected on days 1

and 22 were diluted 100-fold and sera collected on day 37 were diluted 200-fold. Within the 96-well plate, sera were diluted serially down the plate in 2-fold dilutions. The remaining steps of the ELISA protocol followed Hassett *et al*. [18].

Endpoint titers were determined by fitting the OD450 values with a 4-parametic logistic equation as a function of dilution using Microsoft Excel 2011's solver function (Microsoft, Redmond, WA). The cutoff value to be considered a positive signal was based on the average plus 2 times the standard deviation of OD450 values determined from ELISA analyses of serum samples taken from naïve mice. Antibody titers were compared with the nonparametric Mann-Whitney U test using the Minitab (Minitab Inc., State College, PA) statistical software package. Statistical significance was assigned at $p<0.05$.

Pseudovirus neutralization assays. Pseudovirus neutralization assays were used to determine neutralizing antibody titers produced by vaccinated mice[18]. Briefly, various dilutions of mouse sera were added to HPV16, 18 or 31 pseudovirus containing a secreted alkaline phosphatase (SEAP) reporter and incubated on ice for 1 hour to allow for neutralization. This pseudovirus/mouse serum solution was then added to 293TT cells plated in 96-well tissue culture plates, and incubated at 37 °C for 3 days. After incubation, supernatant was collected from cells and assayed for SEAP levels using the Great Escape SEAP Chemiluminescence test kit (Clontech, Mountain View, CA). Plates were read on a multifunctional BioTek plate luminometer at a set glow-endpoint of 0.20 s/well. For a detailed protocol, see (http://home.ccr.cancer.gov/lco/neutralizationassay.htm).
The neutralization titer was defined as the dilution of mouse serum that neutralized 50% of the pseudovirus as determined by SEAP luminescence.

Results

Trivalent vaccine antigen interference. Neutralizing responses against HPV16, 18 and 31 in the trivalent capsomere vaccine formulations and in their respective

monovalent formulations were indistinguishable (Fig. 1a-c). Thus, no discernable interference between antigen types could be observed.

Thermostability of lyophilized monovalent HPV L1 capsomere vaccines. As shown in Figures 1a-c, lyophilized, monovalent formulations of HPV16, 18 and 31 L1 capsomere vaccines retained, or slightly increased, their immunogenicity after incubation for 3 months at 50 °C, eliciting antibody titers essentially equivalent to those generated by their corresponding formulations prior to high-temperature storage. These results were consistent with previously reported results for monovalent formulations of HPV16 LI capsomeres[18]. In contrast, liquid monovalent formulations of HPV16, 18 and 31 L1 capsomere vaccines stored at 50 °C for three months (Fig 2a-c) resulted in significant reduction in antibody responses.

Following the second injection, HPV16, 18 and 31 L1 capsomere formulations adjuvanted with alum or alum and MPLA induced antibody titers that were higher than those generated by their respective adjuvant-free formulations. However, compared to formulations adjuvanted with alum alone, addition of MPLA did not result in further increases in immune response. (Fig. 2a-c).

Trivalent HPV L1 capsomere vaccine thermostability. Trivalent HPV L1 capsomere formulations stored for 3 months at 50 °C elicited high anti-HPV16, 18 and HPV31 L1 antibody titers (Fig. 3) and high levels of neutralizing antibodies (Fig. 4).

Anti-HPV16 L1 antibody titers following administration of adjuvanted trivalent L1 capsomere formulations were similar to those seen for Gardasil®9. For both adjuvanted formulations and Gardasil®9, anti-HPV16 L1 titers measured 22 days after the first injection and prior to the second injection were lower for formulations that had been incubated for three months at 50 °C. However, on Day 37 (two weeks after the second injection), anti-HPV16 antibody titers in response to samples that had been incubated at 50 °C were indistinguishable from those generated against non-incubated control formulations, with the exception of the alum-adjuvanted capsomere formulation. Anti-HPV16 L1 antibody titers measured

on Day 37, two weeks after the second dose of the alum-adjuvanted formulations, decreased somewhat after high temperature storage, but were indistinguishable from responses to the alum plus MPLA and Gardasil®9 vaccine formulations (Fig. 3a). Surprisingly, HPV16 L1 capsomere formulations without added adjuvant generated higher anti-HPV16 antibody titers after the formulations had been stored at 50 °C for three months. Overall, this suggests that three months of incubation at 50 °C did not negatively impact the immunogenicity of HPV16 L1 capsomeres in the trivalent formulation.

Storage for 3 months at 50 °C also had little effect on the anti-HPV18 antibody response to the two adjuvanted trivalent capsomere vaccines and Gardasil®9 (Figure 3b). A slight decrease in anti-HPV18 L1 antibody titers measured two weeks after the second dose was observed after 3 months of storage at 50 °C for the capsomere vaccine that was adjuvanted with both alum and MPLA. In contrast, as was observed for anti-HPV16 antibody titers, anti-HPV18 antibody titers actually increased after storage for 3 months at 50 °C for vaccines formulated without adjuvants.

Anti-HPV31 antibody responses to the trivalent formulations and Gardasil®9 appeared to be somewhat more sensitive to storage at elevated temperature than anti-HPV16 or anti-HPV18 antibody responses. On Day 37, two weeks after the second dose, anti-HPV31 antibody responses generated by samples stored at 50 °C were lower than unincubated samples for trivalent capsomere vaccines adjuvanted with alum alone, and for Gardasil®9 (Figure 3c).

Neutralizing antibody levels (Figure 4a-c) were measured after administration of two doses of Gardasil®9 or trivalent capsomere formulations that had been incubated for three months at 50 °C. Neutralizing titers against HPV16 were similar for Gardasil®9 and both of the adjuvanted trivalent capsomere vaccine formulations. Trivalent capsomere formulations without adjuvant showed noticeably lower HPV16 neutralizing titers. HPV18 neutralizing titers generated in response to Gardasil®9 and the un-adjuvanted trivalent capsomere formulation were lower than those generated by the adjuvanted capsomere vaccines. Anti-

HPV31 neutralizing responses were similar for all of the capsomere vaccine formulations, and were higher than those produced by Gardasil®9.

Discussion

Despite the commercial availability of an efficacious HPV vaccine, cervical cancer remains the second most common cancer among women[36]. In low-resource countries, barriers to HPV vaccination include the high cost of the vaccine, strict cold-chain requirements and the need for booster doses[33, 37, 38]. A thermostable HPV vaccine could improve access in low-resource regions by removing the cold-chain requirement and potentially reducing the cost of vaccination associated with cold-chain maintenance[18]. Previous observations by Hassett *et al.* suggested that thermostable formulations of L1 capsomeres purified after bacterial expression may offer a lower-cost alternative to VLPs used in commercial HPV vaccines[18]. Extending these observations, we prepared an adjuvanted, lyophilized, trivalent L1 capsomere-based HPV vaccine containing HPV types 16, 18, and 31 to assess the efficacy and thermostability of combined capsomere subunit vaccines.

A trivalent HPV vaccine formulation that protects against HPV types 16, 18, and 31 could prevent up to 75% of cervical cancers. However, stabilizing multivalent vaccines may be challenging[35]. Adding multiple antigens to a vaccine can alter the safety and immunogenicity of the previously single-antigen vaccine[39, 40]. The combination of more than one antigen can potentially diminish or enhance the immunogenicity of another in formulation[37]. This immunological interference is unfortunately unpredictable because of multi-factorial interactions within formulations[37]. The antigens also may have competing stability profiles, requiring different optimal formulations (*i.e.,* stabilizing excipient, pH, adjuvant)[41]. The FDA has guidelines for non-inferiority tests for multi-serotype or multi-disease vaccines to demonstrate that antigen combinations do not exhibit negative interference[42-44]. In the current study, antibody responses and pseudovirus neutralizing titers against capsomeres of each HPV type (16, 18, and 31) were equivalent in monovalent and

trivalent formulations. Immunological interference may be less likely in multi-serotype vaccines against one disease than it is for a multi-component vaccine designated to protect against multiple diseases (e.g. tetanus, diphtheria, and pertussis)[39, 44].

A number of different techniques (e.g., lyophilization[45], atomic layer deposition coating[46], spray drying[47], thin film drying[48], entrappping within silica pores[49] and embedding within various polymeric matrices[50] have been used to stabilize vaccines against thermal stresses. For currently licensed vaccines, lyophilization is the most common method used to stabilize vaccines in a solid state; however, lyophilizing adjuvanted vaccines is problematic, and there are no licensed adjuvanted vaccines that are lyophilized and stored as a dried cake. If a vaccine requires an adjuvant it is sometimes co-vialed so that lyophilized antigen and liquid suspensions of adjuvant are prepared in separate vials, thus requiring bedside-mixing prior to administration[24, 51] and adding complexity and cost to vaccination. Adjuvants such as MPLA or GLA (glucopyranosyl lipid A adjuvant) have only recently been shown to be successfully co-lyophilized in the same vial with antigens[23,18, 26, 52]. In the present case, L1 capsomere formulations containing either alum or the alum-MPLA adjuvant system were successfully co-lyophilized and elicited strong immune responses upon reconstitution.

Antibody responses to liquid formulations of capsomeres were greatly reduced after three month of storage at 50 °C. In contrast, responses to lyophilized formulations that contained alum or both alum and MPLA were unchanged or even somewhat increased after incubation for three months at 50 °C. Adsorption to alum or aluminum hydroxyphosphate sulfate adjuvant particles appeared to improve stability at elevated temperatures, since capsomeres formulations without alum suffered the most loss of immunogenicity during storage at elevated temperatures. Consistent with this interpretation, Gardasil-9, a liquid suspension of VLPs adsorbed aluminum hydroxyphosphate sulfate adjuvant particles, retained immunogenicity against HPV16 and HPV18 after the three-month, high temperature incubation.

It appears that the HPV31 antigens were the most temperature-sensitive of the three types tested. When stored at 50 °C, liquid formulations of HPV31 capsomeres lost all ability to generate neutralizing antibodies, even when adsorbed to alum. Similarly, high-temperature incubation of Gardasil-9 resulted in lower antibody responses and lower neutralizing antibody responses for only the HPV31 serotype.

Conclusions

Single-vial lyophilization of HPV capsomeres adsorbed to microparticulate alum adjuvant allows multivalent, capsomere-based HPV vaccines to be stabilized against thermally-induced damage. In tests conducted in mice, overall antibody titers as well as neutralizing antibody titers generated by the lyophilized capsomere vaccines were not affected by incubation at 50 °C for three months. Furthermore, no interference was observed between the HPV16, HPV18 and HPV31 capsomere antigens when co-lyophilized in a single vial. Thus, the lyophilized, multivalent HPV vaccine formulations tested here offer the potential for reduced cold-chain requirements.

Acknowledgements

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.

Conflicts: The University of Colorado owns certain intellectual property rights related to vaccine formulations. TWR and RLG hold equity in VitriVax, Inc., which has licensed some of these intellectual property rights.

Figure 1a

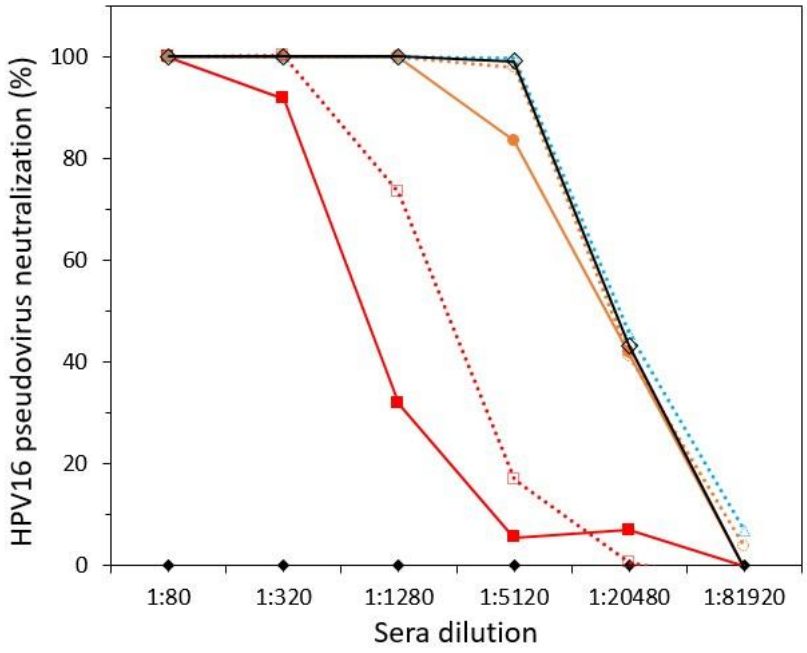

Figure 1b

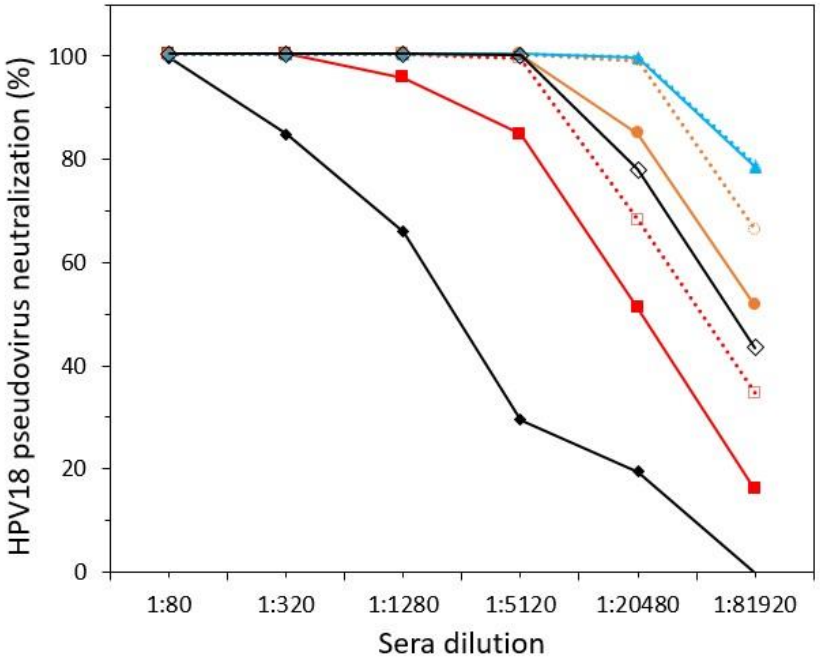

Figure 1c

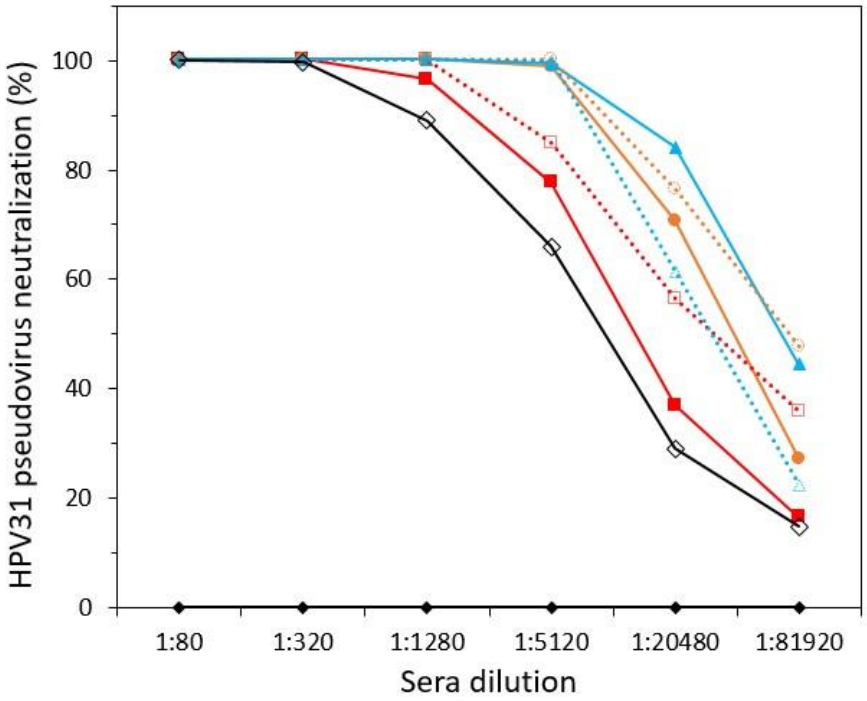

Fig. 1. Comparison of neutralizing antibody responses in monovalent and trivalent vaccines against HPV16 (a), HPV18 (b), and HPV31 (c) as measured by pseudovirus neutralization assays in mice receiving L1 capsomere formulations or Gardasil®9. Mice were vaccinated with capsomere formulations lyophilized without adjuvant (monovalent: red, closed ■ and trivalent: open squares □), lyophilized with alum (monovalent: orange, closed ● and trivalent: open circles ○), and lyophilized with both alum and MPLA (monovalent: closed ▲ and trivalent: open triangles △). Corresponding liquid formulations of monovalent L1 capsomere vaccines contained alum (black, closed diamonds ◆), and can be compared against liquid formulations of Gardasil®9 (black, open diamonds ◇). Mice vaccinated with Gardasil®9 received VLP doses of 6 µg, 4 µg, and 2 µg for HPV types 16, 18, and 31, respectively. Mice received 5 µg of HPV16, HPV18, or HPV31 L1 capsomeres when vaccinated with monovalent formulations, or 5 µg of L1 capsomeres of each HPV type if vaccinated with trivalent formulations. Neutralizing antibodies were measured in serum samples collected on Day 37, 2 weeks after the second injection. Values shown are a geometric mean of n = 5.

Figure 2a

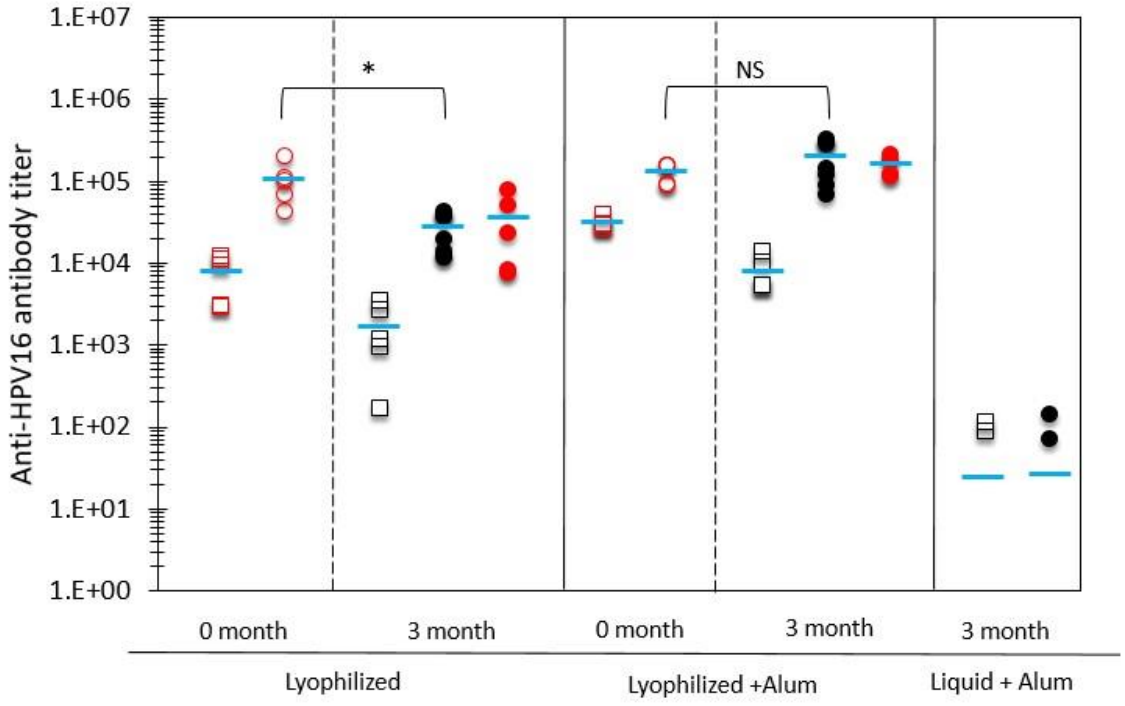

Figure 2b

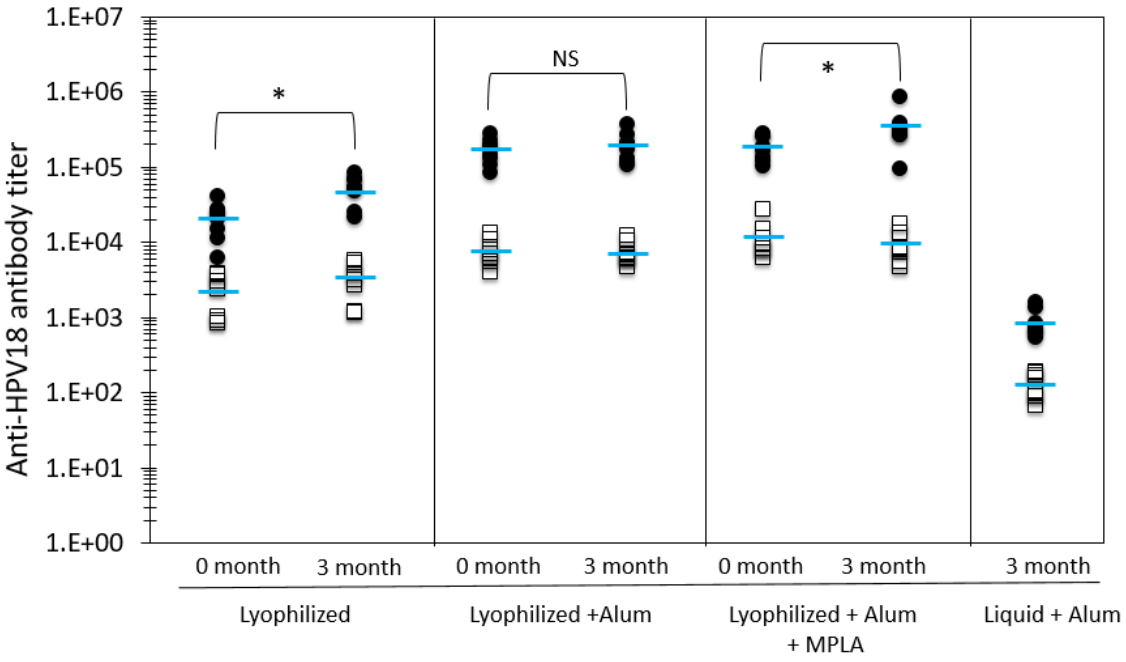
Anti-HPV18 antibody titer
1.E+07
1.E+06
1.E+05
1.E+04
1.E+03
1.E+02
1.E+01
1.E+00
*
NS
*
0 month
3 month
0 month
3 month
0 month
3 month
3 month
Lyophilized
Lyophilized +Alum
Lyophilized + Alum + MPLA
Liquid + Alum

Figure 2c

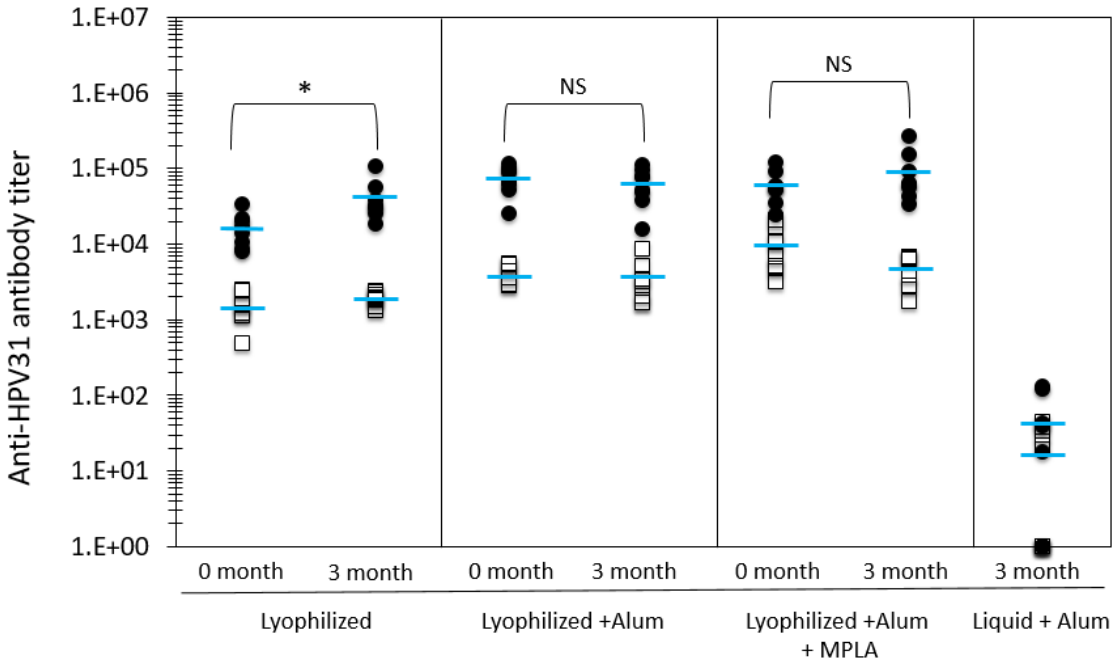
Anti-HPV31 antibody titer
1.E+07
1.E+06
1.E+05
1.E+04
1.E+03
1.E+02
1.E+01
1.E+00
*
NS
NS
0 month
3 month
0 month
3 month
0 month
3 month
3 month
Lyophilized
Lyophilized +Alum
Lyophilized +Alum + MPLA
Liquid + Alum

Fig. 2. Antibody response in mice to monovalent formulations of HPV16 (a), HPV18 (b) and HPV31 (c) as measured by ELISAs. Lyophilized vaccine formulations were reconstituted and administered immediately after preparation ("0 month"), or after 3 months of storage at 50 °C ("3 month"). Each dose contained dose 5 μg L1. Open squares (□) represent antibody titers measured in serum samples collected on Day 22 and solid circles (●) represent the titers measured in serum samples collected on Day 37, two weeks after the second injection. Red markers in (a) represent data from Hassett et al., 2015. Lyophilized vaccines were formulated without any adjuvant, with alum, or with both alum and MPLA. Liquid vaccines were formulated with alum. Significance of differences between antibody responses in serum samples collected on Day 37 as a function of storage conditions were measured by Mann-Whitney U-test; * = $p < 0.05$; NS = no significance.

Figure 3a

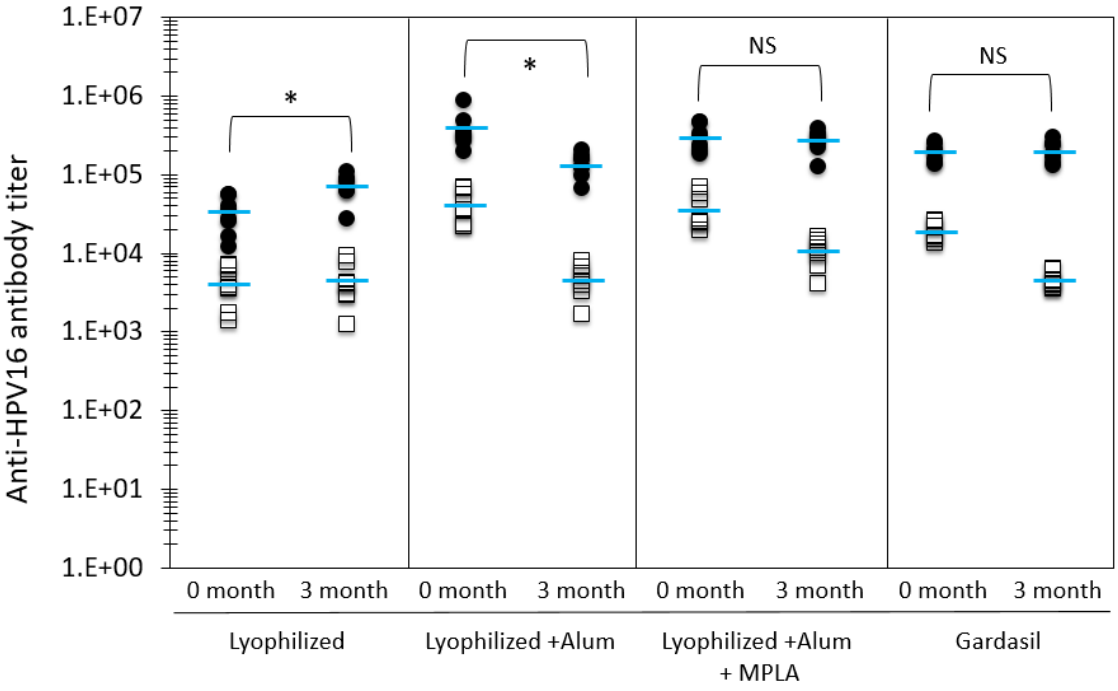

Figure 3b

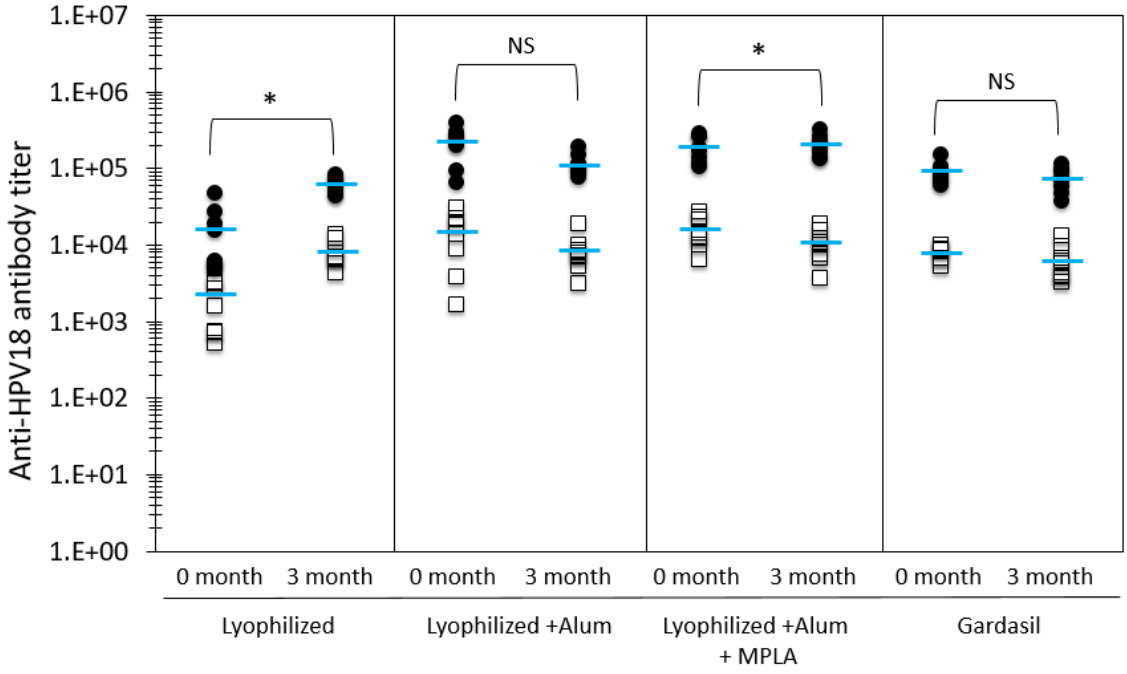

Figure 3c

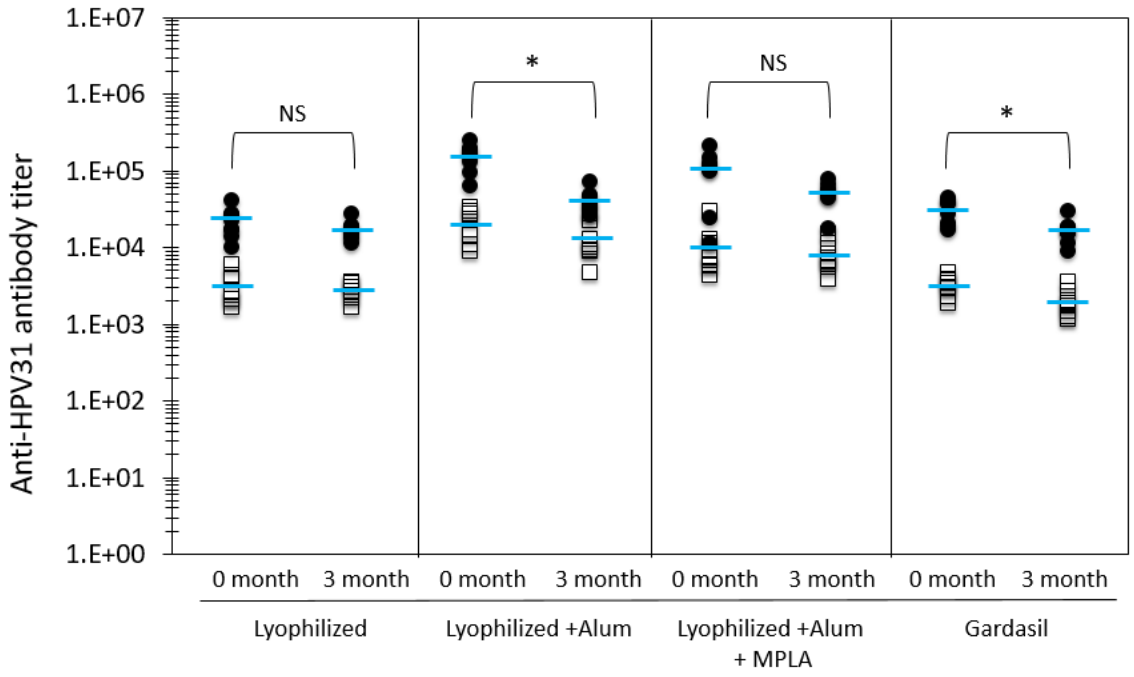

Fig. 3. Antibody response to trivalent, lyophilized HPV L1 capsomere formulations and Gardasil®9 as measured by ELISAs. Lyophilized formulations were reconstituted and administered immediately after preparation ("0 month"), or after 3 months of storage at 50 °C ("3 month"). ELISAs specific for HPV16 (a), HPV18 (b), and HPV31 (c) were used to measure immune responses. Open squares (□) represent antibody titers measured in serum samples collected on Day 22 and solid circles (●) represent the titers measured in serum samples collected on Day 37, two weeks after the second injection. Capsomere vaccines were formulated without any adjuvant, with alum, or with both alum and MPLA and mice received 5 μg of each HPV type per dose. Mice vaccinated with Gardasil®9 received 6, 4, and 2 μg of HPV VLP types 16, 18, and 31 respectively. Significance between storage conditions as measured by Mann-Whitney U-test; * = $p < 0.05$; NS = no significance.

Figure 4a

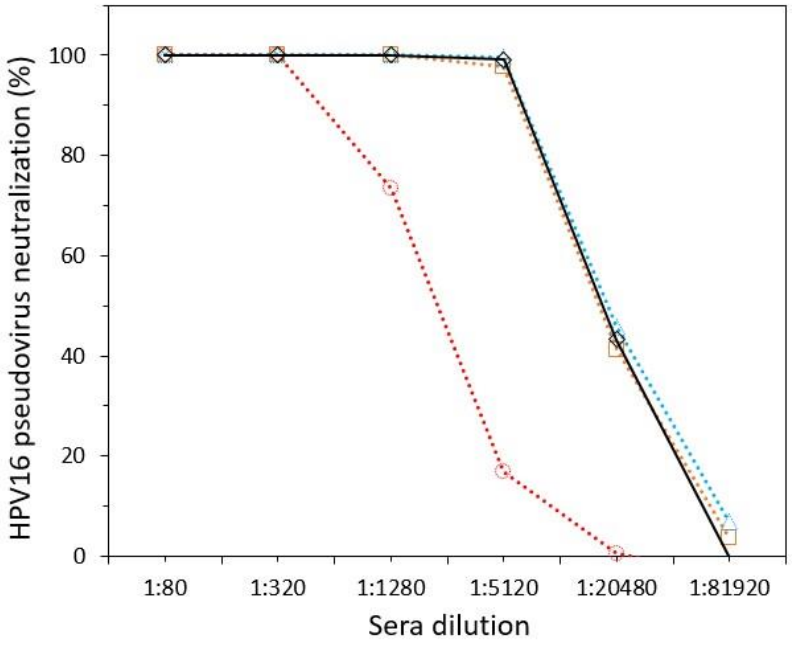
HPV16 pseudovirus neutralization (%)
100
80
60
40
20
0
1:80
1:320
1:1280
1:5120
1:20480
1:81920
Sera dilution

Figure 4b

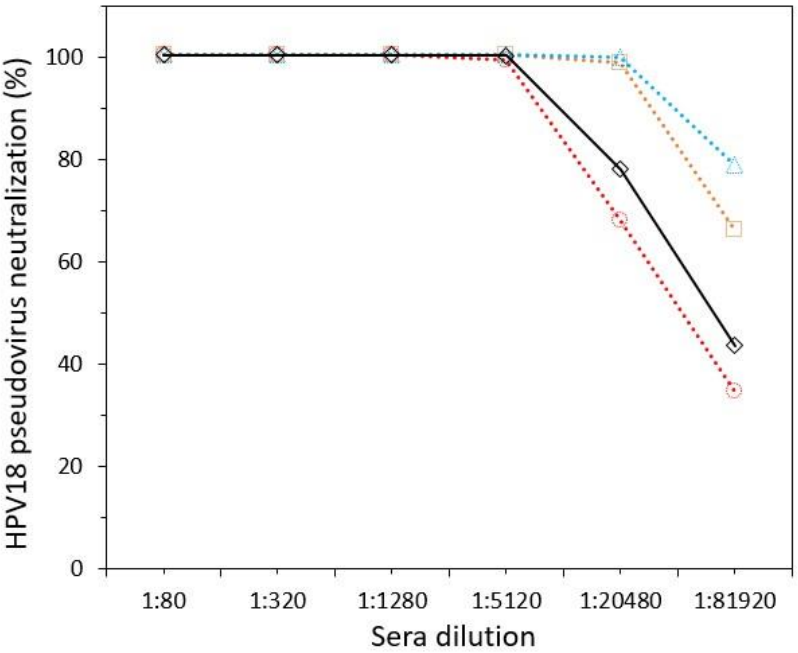
HPV18 pseudovirus neutralization (%)
100
80
60
40
20
0
1:80
1:320
1:1280
1:5120
1:20480
1:81920
Sera dilution

Figure 4c

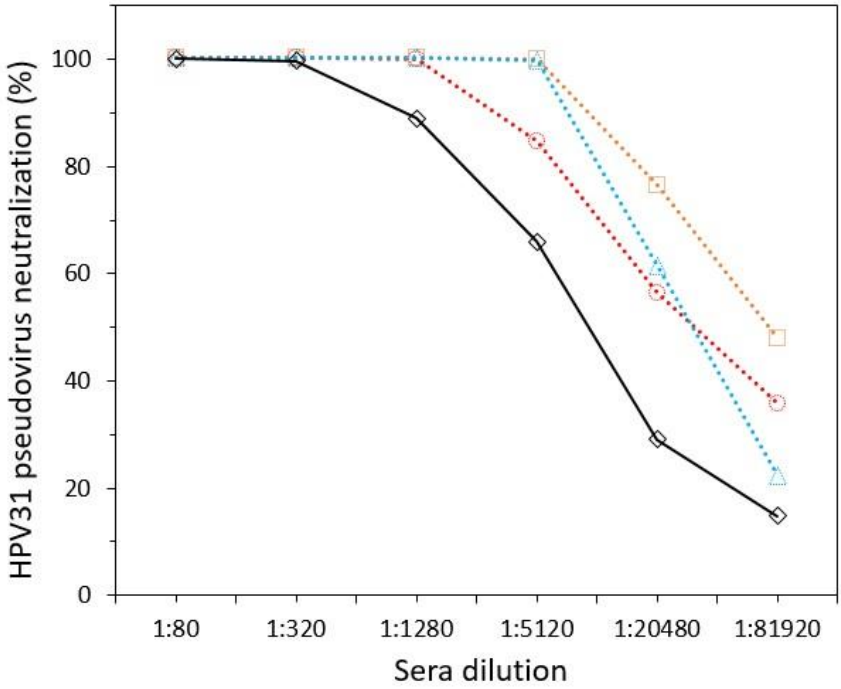

Fig. 4. Neutralizing antibody responses measured on Day 37, 2 weeks after the second injection in mice receiving trivalent, lyophilized HPV L1 capsomere formulations and Gardasil®9, as measured by pseudovirus neutralization. Lyophilized formulations were reconstituted and administered after 3 months of storage at 50 °C at a dose of 5 µg of each HPV type per mouse. Liquid formulations of Gardasil®9 were stored for 3 months at 50 °C and administered at doses of 6, 4, and 2 µg for VLPs of HPV 16, HPV18, and HPV31, respectively. Neutralizing assays specific for HPV16 (a), HPV18 (b), and HPV31 (c) were used measure the neutralizing antibodies made against each HPV type. Lyophilized L1 capsomere vaccines contained no adjuvant (red, open circles ○), alum only (orange, open squares □), alum with MPLA (blue, open triangles △), and were compared with liquid formulations of Gardasil®9 (black, open diamonds ◇). Values shown are a geometric mean of n = 5.

References

1. Kumru, O. S.; Joshi, S. B.; Smith, D. E.; Middaugh, C. R.; Prusik, T.; Volkin, D. B., Vaccine instability in the cold chain: mechanisms, analysis and formulation strategies. *Biologicals* **2014,** *42* (5), 237-59.
2. Chen, D.; Kristensen, D., Opportunities and challenges of developing thermostable vaccines. *Expert Rev Vaccines* **2009,** *8* (5), 547-57.
3. Boros, C. A.; Hanlon, M.; Gold, M. S.; Roberton, D. M., Storage at -3 degrees C for 24 h alters the immunogenicity of pertussis vaccines. *Vaccine* **2001,** *19* (25-26), 3537-42.
4. Chen, X.; Fernando, G. J.; Crichton, M. L.; Flaim, C.; Yukiko, S. R.; Fairmaid, E. J.; Corbett, H. J.; Primiero, C. A.; Ansaldo, A. B.; Frazer, I. H.; Brown, L. E.; Kendall, M. A., Improving the reach of vaccines to low-resource regions, with a needle-free vaccine delivery device and long-term thermostabilization. *J Control Release* **2011,** *152* (3), 349-55.
5. Lee, B. Y.; Cakouros, B. E.; Assi, T. M.; Connor, D. L.; Welling, J.; Kone, S.; Djibo, A.; Wateska, A. R.; Pierre, L.; Brown, S. T., The impact of making vaccines thermostable in Niger's vaccine supply chain. *Vaccine* **2012,** *30* (38), 5637-43.
6. Schlehuber, L. D.; McFadyen, I. J.; Shu, Y.; Carignan, J.; Duprex, W. P.; Forsyth, W. R.; Ho, J. H.; Kitsos, C. M.; Lee, G. Y.; Levinson, D. A.; Lucier, S. C.; Moore, C. B.; Nguyen, N. T.; Ramos, J.; Weinstock, B. A.; Zhang, J.; Monagle, J. A.; Gardner, C. R.; Alvarez, J. C., Towards ambient temperature-stable vaccines: the identification of thermally stabilizing liquid formulations for measles virus using an innovative high-throughput infectivity assay. *Vaccine* **2011,** *29* (31), 5031-9.
7. Bosch, F. X.; Lorincz, A.; Muñoz, N.; Meijer, C. J.; Shah, K. V., The causal relation between human papillomavirus and cervical cancer. *J Clin Pathol* **2002,** *55* (4), 244-65.
8. Bouvard, V.; Baan, R.; Straif, K.; Grosse, Y.; Secretan, B.; El Ghissassi, F.; Benbrahim-Tallaa, L.; Guha, N.; Freeman, C.; Galichet, L.; Cogliano, V.; Group, W. I. A. f. R. o. C. M. W., A review of human carcinogens--Part B: biological agents. *Lancet Oncol* **2009,** *10* (4), 321-2.
9. Walboomers, J. M.; Jacobs, M. V.; Manos, M. M.; Bosch, F. X.; Kummer, J. A.; Shah, K. V.; Snijders, P. J.; Peto, J.; Meijer, C. J.; Muñoz, N., Human papillomavirus is a necessary cause of invasive cervical cancer worldwide. *J Pathol* **1999,** *189* (1), 12-9.
10. Schiller, J. T.; Lowy, D. R., Understanding and learning from the success of prophylactic human papillomavirus vaccines. *Nat Rev Microbiol* **2012,** *10* (10), 681-92.
11. Agosti, J. M.; Goldie, S. J., Introducing HPV vaccine in developing countries - Key challenges and issues. *New England Journal of Medicine* **2007,** *356* (19), 1908-1910.

12. Schauner, S.; Lyon, C., Bivalent HPV Recombinant Vaccine (Cervarix) for the Prevention of Cervical Cancer. *American Family Physician* **2010,** *82* (12), 1541-+.
13. Franco, E. L.; Harper, D. A., Vaccination against human papillomavirus infection: a new paradigm in cervical cancer control. *Vaccine* **2005,** *23* (17-18), 2388-2394.
14. Jemal, A.; Bray, F.; Center, M. M.; Ferlay, J.; Ward, E.; Forman, D., Global Cancer Statistics. *Ca-a Cancer Journal for Clinicians* **2011,** *61* (2), 69-90.
15. Mach, H.; Volkin, D. B.; Troutman, R. D.; Wang, B.; Luo, Z.; Jansen, K. U.; Shi, L., Disassembly and reassembly of yeast-derived recombinant human papillomavirus virus-like particles (HPV VLPs). *Journal of Pharmaceutical Sciences* **2006,** *95* (10), 2195-2206.
16. Shi, L.; Sings, H. L.; Bryan, J. T.; Wang, B.; Wang, Y.; Mach, H.; Kosinski, M.; Washabaugh, M. W.; Sitrin, R.; Barr, E., GARDASIL (R): Prophylactic human papillomavirus vaccine development - From bench top to bed-side. *Clinical Pharmacology & Therapeutics* **2007,** *81* (2), 259-264.
17. Jagu, S.; Kwak, K.; Garcea, R.; Roden, R., Vaccination with multimeric L2 fusion protein and L1 VLP or capsomeres to broaden protection against HPV infection. *Vaccine* **2010,** *28* (28), 4478-4486.
18. Thones, N.; Herreiner, A.; Schadlich, L.; Piuko, K.; Muller, M., A direct comparison of human papillomavirus type 16 L1 particles reveals a lower immunogenicity of capsomeres than viruslike particles with respect to the induced antibody response. *Journal of Virology* **2008,** *82* (11), 5472-5485.
19. Hem, S. L.; White, J. L., Characterization of aluminum hydroxide for use as an adjuvant in parenteral vaccines. *J Parenter Sci Technol* **1984,** *38* (1), 2-10.
20. Zapata, M. I.; Feldkamp, J. R.; Peck, G. E.; White, J. L.; Hem, S. L., Mechanism of freeze-thaw instability of aluminum hydroxycarbonate and magnesium hydroxide gels. *J Pharm Sci* **1984,** *73* (1), 3-8.
21. Maa, Y. F.; Zhao, L.; Payne, L. G.; Chen, D., Stabilization of alum-adjuvanted vaccine dry powder formulations: mechanism and application. *J Pharm Sci* **2003,** *92* (2), 319-32.
22. Clapp, T.; Siebert, P.; Chen, D. X.; Braun, L. J., Vaccines with Aluminum-containing Adjuvants: Optimizing Vaccine Efficacy and Thermal Stability. *Journal of Pharmaceutical Sciences* **2011,** *100* (2), 388-401.
23. Kristensen, D.; Chen, D.; Cummings, R., Vaccine stabilization: research, commercialization, and potential impact. *Vaccine* **2011,** *29* (41), 7122-4.
24. Orr, M. T.; Kramer, R. M.; Barnes, V. L.; Dowling, Q. M.; Desbien, A. L.; Beebe, E. A.; Laurance, J. D.; Fox, C. B.; Reed, S. G.; Coler, R. N.; Vedvick, T. S., Elimination of the cold-chain dependence of a nanoemulsion adjuvanted vaccine against tuberculosis by lyophilization. *Journal of Controlled Release* **2014,** *177*, 20-26.
25. Clausi, A. L.; Merkley, S. A.; Carpenter, J. F.; Randolph, T. W., Inhibition of Aggregation of Aluminum Hydroxide Adjuvant During Freezing and Drying. *J Pharm Sci* **2008**.
26. Hassett, K. J.; Vance, D. J.; Jain, N. K.; Sahni, N.; Rabia, L. A.; Cousins, M. C.; Joshi, S.; Volkin, D. B.; Middaugh, C. R.; Mantis, N. J.; Carpenter, J. F.; Randolph, T. W., Glassy-state stabilization of a dominant negative inhibitor anthrax vaccine

containing aluminum hydroxide and glycopyranoside lipid A adjuvants. *J Pharm Sci* **2015,** *104* (2), 627-39.
27. Hatley, R. H. M.; Blair, J. A., Stabilisation and delivery of labile materials by amorphous carbohydrates and their derivatives. *Journal of Molecular Catalysis B-Enzymatic* **1999,** *7* (1-4), 11-19.
28. Carpenter, J. F.; Chang, B. S.; Garzon-Rodriguez, W.; Randolph, T. W., Rational design of stable lyophilized protein formulations: theory and practice. *Pharm Biotechnol* **2002,** *13*, 109-33.
29. Hassett, K. J.; Cousins, M. C.; Rabia, L. A.; Chadwick, C. M.; O'Hara, J. M.; Nandi, P.; Brey, R. N.; Mantis, N. J.; Carpenter, J. F.; Randolph, T. W., Stabilization of a recombinant ricin toxin A subunit vaccine through lyophilization. *Eur J Pharm Biopharm* **2013**.
30. Roy, S.; Henderson, I.; Nayar, R.; Randolph, T. W.; Carpenter, J. F., Effect of pH on Stability of Recombinant Botulinum Serotype A Vaccine in Aqueous Solution and During Storage of Freeze-Dried Formulations. *Journal Of Pharmaceutical Sciences* **2008,** *97* (12), 5132-5146.
31. Chisholm, C. F.; Kang, T. J.; Dong, M.; Lewis, K.; Namekar, M.; Lehrer, A. T.; Randolph, T. W., Thermostable Ebola virus vaccine formulations lyophilized in the presence of aluminum hydroxide. *European Journal of Pharmaceutics and Biopharmaceutics* **2019,** *136*, 213-220.
32. Hassett, K. J.; Meinerz, N. M.; Semmelmann, F.; Cousins, M. C.; Garcea, R. L.; Randolph, T. W., Development of a highly thermostable, adjuvanted human papillomavirus vaccine. *Eur J Pharm Biopharm* **2015,** *94*, 220-8.
33. Sankaranarayanan, R., HPV vaccination: the promise & problems. *Indian Journal of Medical Research* **2009,** *130* (3), 322-326.
34. Smith, J. S.; Lindsay, L.; Hoots, B.; Keys, J.; Franceschi, S.; Winer, R.; Clifford, G. M., Human papillomavirus type distribution in invasive cervical cancer and high-grade cervical lesions: A meta-analysis update. *International Journal of Cancer* **2007,** *121* (3), 621-632.
35. Mariani, L.; Monfulleda, N.; Alemany, L.; Vizza, E.; Marandino, F.; Vocaturo, A.; Benevolo, M.; Quiros, B.; Lloveras, B.; Klaustermeier, J. E.; Quint, W.; de Sanjose, S.; Bosch, F. X., Human Papilloma Virus prevalence and type-specific relative contribution in invasive cervical cancer specimens from Italy. *Bmc Cancer* **2010,** *10*.
36. Forman, D.; de Martel, C.; Lacey, C. J.; Soerjomataram, I.; Lortet-Tieulent, J.; Bruni, L.; Vignat, J.; Ferlay, J.; Bray, F.; Plummer, M.; Franceschi, S., Global Burden of Human Papillomavirus and Related Diseases. *Vaccine* **2012,** *30*, F12-F23.
37. Arbyn, M.; Castellsague, X.; de Sanjose, S.; Bruni, L.; Saraiya, M.; Bray, F.; Ferlay, J., Worldwide burden of cervical cancer in 2008. *Annals of Oncology* **2011,** *22* (12), 2675-2686.
38. Gersch, E.; Gissmann, L.; Garcea, R., New approaches to prophylactic human papillomavirus vaccines for cervical cancer prevention. *Antiviral Therapy* **2012,** *17* (3), 425-434.
39. Vidor, E., The nature and consequences of intra- and inter-vaccine interference. *Journal of Comparative Pathology* **2007,** *137*, S62-S66.
40. Saul, A.; Fay, M. P., Human Immunity and the Design of Multi-Component, Single Target Vaccines. *Plos One* **2007,** *2* (9).

41. Borde, A.; Ekman, A.; Larsson, A.; Carlin, N.; Holmgren, J.; Tobias, J., Preparation and preclinical evaluation of a freeze-dried formulation of a novel combined multivalent whole-cell/B-subunit oral vaccine against enterotoxigenic Escherichia coli diarrhea. *European Journal of Pharmaceutics and Biopharmaceutics* **2016,** *108*, 18-24.
42. Goldenthal, K. L.; Falk, L. A.; Ball, L.; Geber, A., Prelicensure evaluation of combination vaccines. *Clinical Infectious Diseases* **2001,** *33*, S267-S273.
43. Guidance for Industry for the Evaluation of Combination Vaccines for Preventable Diseases: Production, Testing, and Clinical Studies. United States Food and Drug Administration: 1997.
44. Capiau, C.; Poolman, J.; Hoet, B.; Bogaerts, H.; Andre, F., Development and clinical testing of multivalent vaccines based on a diphtheria-tetanus-acellular pertussis vaccine: difficulties encountered and lessons learned. *Vaccine* **2003,** *21* (19-20), 2273-2287.
45. Shearwin, K. E.; Winzor, D. J., Allowance For Thermodynamic Nonideality and Donnan Effects in Binding-Studies - Activity-Coefficients of Charged Ligands in the Presence of Albumin. *Biophysical Chemistry* **1990,** *36* (3), 235-243.
46. Garcea, R. L.; Meinerz, N. M.; Dong, M.; Funke, H.; Ghazvini, S.; Randolph, T. W., Single-administration, thermostable human papillomavirus vaccines prepared with atomic layer deposition technology. *Npj Vaccines* **2020,** *5* (1).
47. Preston, K. B.; Randolph, T. W., Stability of lyophilized and spray dried vaccine formulations. *Adv Drug Deliv Rev* **2021,** *171*, 50-61.
48. Bajrovic, I.; Schafer, S. C.; Romanovicz, D. K.; Croyle, M. A., Novel technology for storage and distribution of live vaccines and other biological medicines at ambient temperature. *Science Advances* **2020,** *6* (10).
49. Wang, G. C.; Wang, H. J.; Zhou, H. Y.; Nian, Q. G.; Song, Z. Y.; Deng, Y. Q.; Wang, X. Y.; Zhu, S. Y.; Li, X. F.; Qin, C. F.; Tang, R. K., Hydrated Silica Exterior Produced by Biomimetic Silicification Confers Viral Vaccine Heat-Resistance. *Acs Nano* **2015,** *9* (1), 799-808.
50. Guo, S. H.; Fu, D. W.; Utupova, A.; Sun, D. J.; Zhou, M.; Jin, Z.; Zhao, K., Applications of polymer-based nanoparticles in vaccine field. *Nanotechnology Reviews* **2019,** *8* (1), 143-155.
51. Iyer, V.; Cayatte, C.; Marshall, J. D.; Sun, J.; Schneider-Ohrum, K.; Maynard, S. K.; Rajani, G. M.; Bennett, A. S.; Remmele, R. L.; Bishop, S. M.; McCarthy, M. P.; Muralidhara, B. K., Feasibility of Freeze-Drying Oil-in-Water Emulsion Adjuvants and Subunit Proteins to Enable Single-Vial Vaccine Drug Products. *Journal of Pharmaceutical Sciences* **2017,** *106* (6), 1490-1498.
52. Fox, C. B.; Carter, D.; Kramer, R. M.; Beckmann, A. M.; Reed, S. G., Current Status of Toll-Like Receptor 4 Ligand Vaccine Adjuvants. *Immunopotentiators in Modern Vaccines, 2nd Edition* **2017**, 105-127.