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Oral immunization with bacteriophage MS2-L2 VLPs protects against oral and genital infection with multiple HPV types associated with head & neck cancers and cervical cancer

Lukai Zhai[a], Rashi Yadav[a], Nitesh K. Kunda[b,c], Dana Anderson[a], Elizabeth Bruckner[d], Elliott K. Miller[b], Rupsa Basu[a], Pavan Muttil[b], Ebenezer Tumban[a,*]

[a] Department of Biological Sciences, Michigan Technological University, Houghton, MI 49931, USA
[b] Department of Pharmaceutical Sciences, College of Pharmacy, University of New Mexico, Albuquerque, NM 87131, USA
[c] Department of Pharmaceutical Sciences, College of Pharmacy and Health Sciences, St. John's University, Jamaica, NY 11439, USA
[d] Department of Chemical Engineering, Michigan Technological University, Houghton, MI 49931, USA

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ABSTRACT

Human papillomaviruses (HPVs) are the most common sexually transmitted infections. HPVs are transmitted through anogenital sex or oral sex. Anogenital transmission/infection is associated with anogenital cancers and genital warts while oral transmission/infection is associated with head and neck cancers (HNCs) including recurrent respiratory papillomatosis. Current HPV vaccines protect against HPV types associated with ~90% of cervical cancers and are expected to protect against a percentage of HNCs. However, only a few studies have assessed the efficacy of current vaccines against oral HPV infections. We had previously developed a mixed MS2-L2 candidate HPV vaccine based on bacteriophage MS2 virus-like particles (VLPs). The mixed MS2-L2 VLPs consisted of a mixture of two MS2-L2 VLPs displaying: i) a concatemer of L2 peptide (epitope 20–31) from HPV31 & L2 peptide (epitope 17–31) from HPV16 and ii) a consensus L2 peptide representing epitope 69–86. The mixed MS2-L2 VLPs neutralized/protected mice against six HPV types associated with ~87% of cervical cancer. Here, we show that the mixed MS2-L2 VLPs can protect mice against additional HPV types; at the genital region, the VLPs protect against HPV53, 56, 11 and at the oral region, the VLPs protect against HPV16, 35, 39, 52, and 58. Thus, mixed MS2-L2 VLPs protect against eleven oncogenic HPV types associated with ~95% of cervical cancer. The VLPs also have the potential to protect, orally, against the same oncogenic HPVs, associated with ~99% of HNCs, including HPV11, which is associated with up to 32% of recurrent respiratory papillomatosis. Moreover, mixed MS2-L2 VLPs are thermostable at room temperature for up to 60 days after spray-freeze drying and they are protective against oral HPV infection.

1. Introduction

Human papillomaviruses (HPVs) are non-enveloped DNA viruses with more than 220 different types identified to date (Burk et al., 2013; Chen et al., 2018; Karolinska, 2018). Approximately, 40 of these HPV types are transmitted sexually through anogenital-to-anogenital sex or anogenital-to-oral sex (Garland et al., 2018; Shah et al., 2017), and 19 HPV types can cause cancers. Anogenital HPV infections are associated with cervical cancer, vaginal cancer, penile cancer, anal cancer, and genital warts (Berman and Schiller, 2017; Boscolo-Rizzo et al., 2013; Crow, 2012; Leslie and Kumar, 2018; Zhai and Tumban, 2016) whereas oral HPV infections are associated with head and neck cancers (HNCs; oral, oropharyngeal, laryngeal cancers, etc.) including recurrent respiratory papillomatosis (RRP) (Chaturvedi et al., 2011; D'Souza et al., 2016; Fortes et al., 2017; Gelwan et al., 2017; Sanchez et al., 2013). Twenty-five percent of HNCs are caused by HPVs (Kreimer et al., 2005); HPV type 16 is associated with more than 70% of HPV-associated HNCs (HPV + HNCs). HPV18 is associated with 14–17% of HPV + HNCs while the remaining HPV + HNC cases are caused by other oncogenic HPV types (31, 33, 35, 45, etc.) (Castellsague et al., 2016; D'Souza et al., 2016; de Martel et al., 2017; Ndiaye et al., 2014). The prevalence of HPV + HNC varies from one geographical region to another; 21.1–40.8% in Europe, ~32.4% in Asia, 24.3–26.3% in North America, 3.7–21.1% in Central & South American, and 1.0–2.5% in Africa (de Martel et al., 2017; Ndiaye et al., 2014). Two prophylactic vaccines (Gardasil-9 and Cervarix; Gardail-4 has been discontinued) are

* Corresponding author.
E-mail address: etumban@mtu.edu (E. Tumban).

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currently being used to protect against HPV infections (FDA, 2018a, 2018b; Zhai and Tumban, 2016). Amongst these vaccines, Gardasil-9 offers the broadest level of protection against HPV types (HPV16, 18, 31, 33, 45, 52, 58) that cause ~90% of cervical cancers worldwide and HPV types (HPV6 and 11) that cause 90% of genital warts/RRP. Despite the broadness of protection offered by Gardasil-9 vaccine, vaccinated individuals are still advised to continue cervical cancer screening to make sure they are not infected with cancer-causing HPV types not included in the vaccine. HPV types (e.g. HPV35, 39, 53, 56, etc.) associated with ~10% of cervical cancer not protected by current vaccines are significantly important especially if this involves patients infected with human immunodeficiency virus (HIV) or are suffering from acquired immunodeficiency syndrome (AIDS). Additionally, there is no data on the efficacy of Gardasil-9 vaccine against oral HPV infections.

To enhance the spectrum of protection and to circumvent these problems, we developed, in a previous study (Zhai et al., 2017), two bacteriophage MS2 VLPs: i) MS2-31L2/16L2 VLP displaying a concatemer of HPV31 L2 (amino acid 20–31) & HPV16 L2 (amino acid 17–31) on its surface; ii) MS2-consL2(69–86) VLP displaying a consensus sequence (amino acid 69–86) derived from the alignment of 19 cancer-causing HPV types and 4 warts-causing HPV types. L2 (the minor capsid protein) is one of the two capsid proteins of HPV; L2, especially the N-terminus, is highly conserved among HPV types and is a target for next-generation HPV vaccines (Gambhira et al., 2007; Kondo et al., 2007; Schellenbacher et al., 2017; Tumban et al., 2012). In Zhai et al., 2017, we showed that mice immunized, intramuscularly, with a mixture of the VLPs, MS2-31L2/16L2 and MS2-consL2(69–86), elicited high-titer ($> 10^4$) IgG antibodies and protected the mice from vaginal challenge with HPV pseudoviruses representing HPV16, 18, 31, 45, and HPV33 at levels similar to Gardasil-9; suboptimal protection was observed against HPV58 (Zhai et al., 2017); these six HPV types are associated with ~87% of cervical cancer and ~90% of HPV + HNCs. To build on that study, we assessed whether oral immunization with the mixed MS2-L2 VLPs in the presence of two mucosal adjuvants (cholera toxin and monophosphoryl lipid A) can protect mice from oral and/or vaginal infection with additional HPV pseudovirus types (HPV35, 39, 52, 53 and HPV56) associated with ~8.9% of HPV + HNC and ~8.0% of cervical cancer; oral immunization (including other mucosal approaches) with cholera toxin or monophosphoryl lipid A has been shown to elicit protective mucosal and systemic immune responses (Baldridge et al., 2000; Childers et al., 2000; Cuburu et al., 2009; Maseda et al., 2018). We also assessed whether the mixed VLPs can be developed into a heat-stable dry powder formulation with the two mucosal adjuvants; we assessed the thermostability/immunogenicity of the dry powder VLPs stored at room temperature for 60 days.

2. Materials and methods

2.1. Production of MS2-L2 VLPs

Plasmids encoding recombinant MS2-L2 proteins – MS2 coat protein with an insertion of a concatemer of HPV L2 epitope containing amino acids (aa) 20–31 from HPV31 L2 and aa 17–31 from HPV16 L2 (MS2-31L2/16L2) or MS2 coat protein with an insertion of a consensus HPV L2 epitope [MS2-consL2(69–86)] - were previously described (Zhai et al., 2017). The plasmids were used to transform C41 *Escherichia coli* cells. Protein expression and VLPs purification were conducted as previously described (Zhai et al., 2017). Briefly, transformed C41 bacterial culture was induced with 0.5 mM Isopropyl β-D-1-thiogalactopyranoside for 3 h and the bacteria were lysed with 0.2% lysozyme solution [for MS2-consL2(69–86) VLPs] or 10 mM Borax (for MS2-31L2/16L2 VLPs). Soluble VLPs were precipitated with 50% ammonium sulfate and purified using Sepharose cross-linked-4B columns.

2.2. Spray-freeze drying of VLPs into dry powder formulation

Equal concentrations of MS2-31L2/16L2 VLPs and MS2-consL2(69–86) VLPs were mixed together to obtain mixed MS2-L2 VLPs. Half of the mixed MS2-L2 VLPs (100 μg) was further mixed with 2 μg each of the mucosal adjuvants, cholera toxin (CT) and monophosphoryl lipid A (MPLA); control MS2 VLPs were mixed with the same concentration of adjuvants. The mixed MS2-L2 VLPs and MS2 VLPs, with or without mucosal adjuvants, were added to a 3% w/v excipient solution [Mannitol (M, 75% w/w), Trehalose (T, 7.5% w/w), Dextran (D, 2.5% w/w), and L-Leucine (L, 15% w/w); MTDL]. The VLPs were added at a concentration of 8% w/w to the MTDL excipients and the VLPs-excipients were then spray-freeze dried (SFD) in two steps using a method adapted from Tonnis et al. (2014) and Ali et al., 2014 (Ali and Lamprecht, 2014; Tonnis et al., 2014). In the first step, the VLPs-excipients suspensions were sprayed into a stainless-steel container filled partially with liquid nitrogen. This was achieved by using a two-fluid nozzle (0.7 mm) which is part of a Büchi B-290 mini spray-dryer; the spraying operating conditions were as following: nitrogen flow between 10 and 15 mm, and a VLPs-excipients feed rate of ~4 mL/min. Subsequently, the liquid nitrogen in the steel container was allowed to evaporate and the frozen droplets were transferred into a lyophilizer (FreeZone® Triad™ Freeze Dry system, Model 74000 Series) for freeze-drying (the second step). Freeze-drying was conducted under the following conditions: pre-freeze for 3 h at −80 °C, primary drying at −10 °C for 24 h with a ramp of 0.25 °C/min, followed by secondary drying at 15 °C for 48 h with a ramp of 0.25 °C/min, and vacuum pressure of 1.51 mBar. All SFD products were collected in glass scintillation vials and were stored under refrigeration until further use.

2.3. Assessing the thermostability of SFD VLPs

Spray-freeze dried VLP powders were loaded into capsules, transferred to amber-colored Wheaton glass bottles and sealed with 20 mm butyl septa caps. The bottles were then purged with nitrogen gas and crimped with tear-away aluminum seals using a vial seal crimper (Kunda et al., 2016). The sealed bottles were then stored at 4 °C or room temperature for 60 days. The powders were then reconstituted in 1X phosphate buffered saline (PBS) and the integrity of VLPs was assessed, in comparison to non-spray-freeze dried VLPs, by transmission electron microscopy (TEM; 30,000X magnification). The immunogenicity of the reconstituted VLPs was assessed by immunizing mice as described below.

2.4. Immunization of mice and assessing antibody responses

All animal work was conducted following Michigan Technological University Institutional Animal Care and Use Committee guidelines (Protocol number L0264). Groups of female balb/c mice (4–6 mice per group) were immunized thrice orally, by placing VLPs underneath the tongue or by injecting into the buccal region, at two-week intervals with freshly prepared (non-spray-freeze dried) mixed MS2-L2 VLPs [50 μg each of MS2-31L2/16L2 VLPs and MS2-consL2(69–86)] mixed with either 2 μg of CT, 2 μg of MPLA, a combination of the two adjuvants (same concentrations), or without adjuvants. Other groups of mice were immunized with control MS2 VLPs and the same adjuvant combinations for comparison. In all immunizations, 5 μg of Gardasil-9 was used as controls administered intramuscularly because it does not contain mucosal adjuvants.

To assess the immunogenicity of the spray-freeze dried VLPs, 100 μg of the reconstituted SFD mixed MS2-L2 or SFD control MS2 VLPs (all SFD with CT and MPLA adjuvants) were used to immunize mice as described above (same route and schedule). Other groups of mice were immunized with the same concentration of reconstituted spray-freeze dried VLPs but with additional 2 μg each of fresh liquid CT and MPLA adjuvants; the latter was to assess whether the activity of the adjuvants

were inactivated during the SFD process. The VLPs that were SFD without the adjuvants were immunized (at same concentration) with 2 μg each of fresh liquid CT and MPLA adjuvants.

In all immunizations, sera and saliva were collected two weeks after the last immunizations. IgG antibody titers in sera were conducted by peptide-ELISA as previously described (Tumban et al., 2011). Briefly, 96-well plates were coated, overnight, with 500 ng HPV16 L2 peptide conjugated to streptavidin. The plates were blocked for two hours with 0.5% non-fat milk in 1X PBS. The plates were then incubated with 4-fold serial dilutions of serum for two hours. Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (1:5000 dilution) antibodies were added to the plates for one hour. The wells were washed and developed with 3, 3′, 5, 5′-Tetramethylbenzidine (TMB) and stopped with 1 M hydrogen chloride solution. Antibody titers were determined (at optical density 450) as the reciprocal of highest sera dilutions at which reactivity of experimental sera was at least twice that of control sera. IgA antibody titers in saliva were determined also by peptide-ELISA with 100 μL saliva samples as primary antibodies. HRP-conjugated goat anti-mouse IgA (1:500) antibodies were added for one hour and the plates were developed as described above except without the stop solution. IgA antibodies were determined by comparing the optical density (405 nm) values of experimental group to those of control group.

2.5. Oral and vaginal infection with HPV pseudoviruses (PsVs)

HPVs pseudoviruses representing PsVs 11, 16, 35, 39, 52, 53, 56 and 58 were expressed and purified by cesium chloride gradient ultracentrifugation as previously described (Tumban et al., 2012). The PsVs encode a reporter plasmid that expresses green fluorescence protein and luciferase. For vaginal infection, immunized mice were treated with Depo-provera and PsVs (11, 16, 53, and 56) infection was conducted as previously described (Zhai et al., 2017). For oral infection, immunized mice were injected at the buccal region with PsVs (16, 35, 39, 52 or 58); 1–7 × 10^6 infectious unit (IU) of PsVs were used in all infections. Forty-eight hours post-infection, mice that were infected orally were injected in the buccal region with 1 mg of luciferin, while those that were infected vaginally were instilled vaginally with 0.4 mg of luciferin. In both cases, the mice were imaged using IVIS Spectrum at one-minute exposure. Average radiance (p/s/cm^2/sr) was determined by drawing equally sized regions of interests surrounding the site of PsV instillation.

2.6. Statistical analysis

Statistical analyses for ELISAs and of HPV PsV challenge studies were done using unpaired two-tailed *t*-test and unpaired one-tailed *t*-test, respectively.

3. Results

3.1. Buccal immunization with mixed MS2-L2 VLPs plus mucosal adjuvants elicits protective immune responses at the vaginal and oral regions

To assess which delivery method of oral immunization will elicit immune responses, mice were immunized by placing freshly prepared (non-spray-freeze dried) mixed MS2-L2 VLPs with/without CT and MPLA in the floor of the mouth or by injecting the buccal area of the mouth with the VLPs (with/without CT and MPLA). We then assessed IgG and IgA antibody levels in sera and saliva, respectively. Mice immunized by injecting the floor of the mouth with the VLPs elicited high-titer IgG antibodies in the sera (Fig. 1A) but no detectable IgA in saliva (data not shown). Mice immunized with the mixed MS2-L2 VLPs in the presence of the two adjuvant combinations (mixed MS2-L2 VLPs + CT/MPLA) elicited higher IgG titers ($p < 0.0001$) compared to mice immunized with the same VLPs but with only one of the adjuvants (especially mixed MS2-L2 VLPs + MPLA; $p = 0.0140$). Mice immunized by placing the VLPs in the floor of the mouth did not elicit immune response in the sera or in the saliva (data not shown). Thus, all subsequent immunization studies were conducted by injecting the buccal region of the mouth and with the two-adjuvant combinations (CT/MPLA).

To assess if the immune responses elicited by buccal immunization were protective, we challenged the mice vaginally, using a well-characterized method for HPV infection (Roberts et al., 2007; Tumban et al., 2011). As shown in Fig. 1B, mice immunized with mixed MS2-L2 VLPs with CT/MPLA showed better levels of protection ($p < 0.0001$) from HPV PsV16 infection compared to mice immunized with only one of the two adjuvants (especially those immunized with MPLA; $p = 0.0072$). To test if buccal immunization with mixed MS2-L2 VLPs offered cross-protection against other HPV PsVs, vaccinated mice were vaginally infected with high-risk HPV PsV53 and PsV56, which are not included in Gardasil-9 vaccine, and with low-risk HPV PsV11. Mice immunized with mixed MS2-L2 VLPs offered complete protection against PsV53 and PsV56 while Gargasil-9 offered partial protection against PsV56 but no protection against PsV53 (Fig. 1C); both mixed MS2-L2 VLPs and Gardasil-9 elicited protective responses against PsV11 (Fig. 1C).

Having demonstrated that buccal immunization protected mice from vaginal infection by HPV PsVs 11, 16, 53, and 56, we decided to assess if buccal immunization can protect mice from oral infection with PsV16 and other PsVs (35, 39, 52 and 58). In Fig. 2, buccal immunization with mixed MS2-L2 VLPs + CT/MPLA protected mice from oral infection with PsVs 16, 35, 39, 52, and 58. While mixed MS2-L2 VLPs and Gardasil-9 (immunized intramuscularly) offered similar levels of protection against PsV16 and PsV52, Gardasil-9 offered better protection against PsV58 and mixed MS2-L2 VLPs offered best protection against PsV35 and PsV39 (Fig. 2B).

3.2. Mixed MS2-L2 VLPs can be SFD without a mixture of cholera toxin/MPLA adjuvants

To assess if mixed MS2-L2 VLPs can be SFD into a product that is thermostable at room temperature, the VLPs with or without CT/MPLA were SFD with stabilizing excipients and the integrity of the VLPs was assessed by TEM. As a control, MS2 VLPs with same adjuvants and excipients were also SFD. While mixed MS2-L2 VLPs or MS2 VLPs in the absence of adjuvants were successfully SFD (based on the integrity of the VLPs after reconstitution of the powder in PBS buffer in comparison to non-SFD VLPs), the VLPs could not be SFD with the adjuvants (Fig. 3). SFD with adjuvants caused the VLPs to agglomerate (Fig. 3A). To assess whether the SFD VLPs (those that did not agglomerate and those that agglomerated) were still immunogenic, we immunized mice (in buccal region) with the VLPs with/without additional CT/MPLA adjuvants. Mixed MS2-L2 VLPs SFD without adjuvant, but adjuvant combinations were added (+CT/MPLA) just prior to immunization were highly immunogenic compared to the VLPs [mixed MS2-L2 VLPs (CT/MPLA)] that were SFD together with the adjuvant combinations (Fig. 4A). The addition of more adjuvant (+CT/MPLA) to the latter [mixed MS2-L2 VLPs (CT/MPLA)] slightly enhanced the immunogenicity of the VLPs but this was not significant. The mice immunized with mixed MS2-L2 VLPs + CT/MPLA were significantly protected from vaginal infection with HPV PsV16 while mice immunized with mixed MS2-L2 VLPs (CT/MPLA) + CT/MPLA elicited suboptimal protection against PsV16 infection (Fig. 4B). Also, the group of mice (SFD mixed MS2-L2 VLPs + CT/MPLA-immunized; Fig. 4B) that showed complete protection from vaginal infection with PsV16 was completely protected from oral infection with the same PsV type (Fig. 4C).

3.3. SFD mixed VLPs are thermostable at room temperature for up to 60 days and elicit protective responses

To evaluate if spray-freeze dried VLPs are thermostable, the VLPs

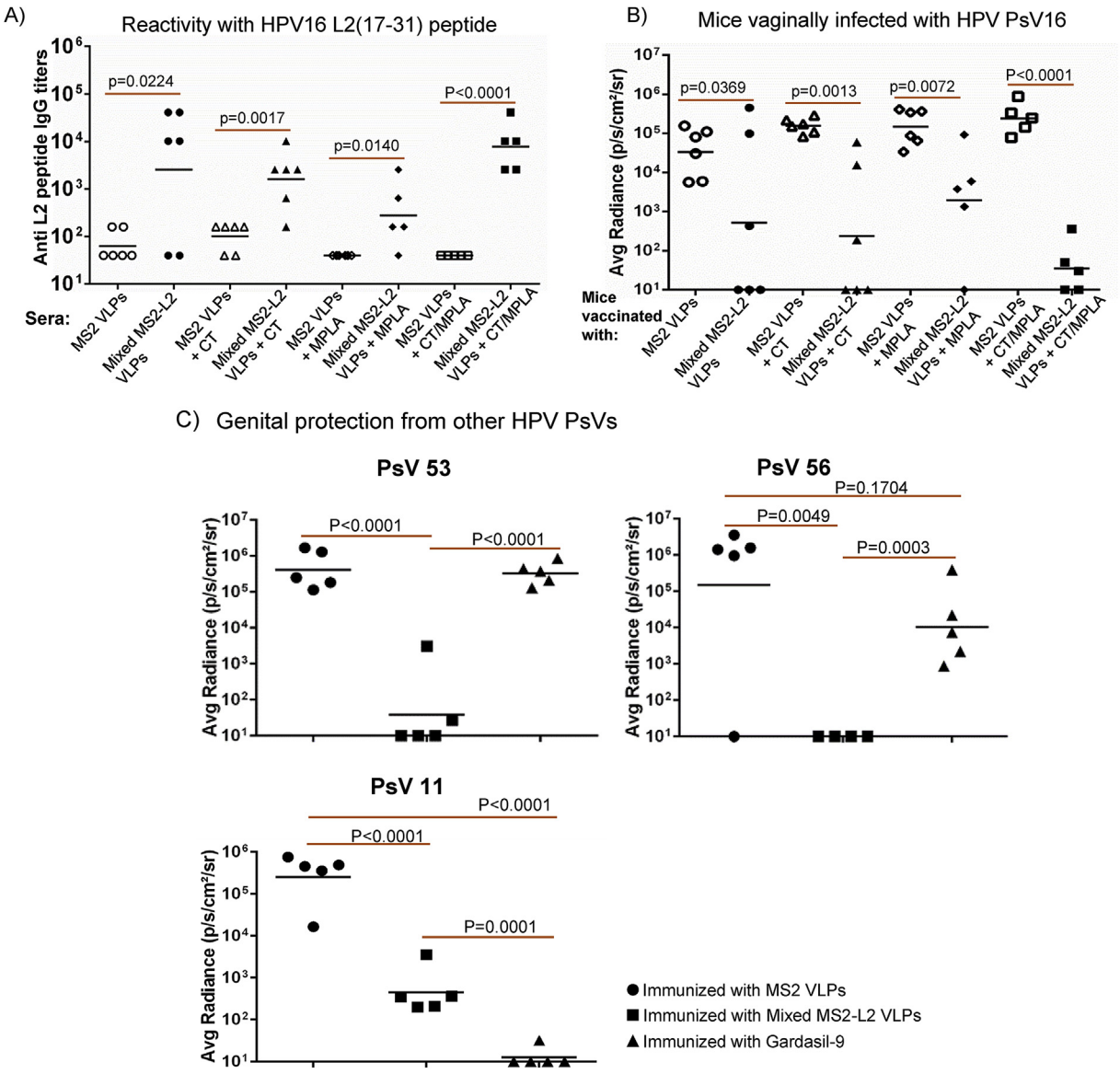

Fig. 1. Immunogenicity of mixed MS2-L2 VLPs with/without mucosal adjuvants (CT or MPLA) and protection from PsV16, 53, 56 and 11. Mice were immunized (buccal injection) thrice with 100 μg of mixed MS2-L2 VLPs or control MS2 VLPs with/without CT, MPLA or with/out the two-adjuvant combinations at two-week intervals. Sera were collected two weeks after the last immunization. A) Anti-L2 peptide IgG titers in sera were determined by end-point dilution ELISA using 16L2 (17–31) peptide as the target peptide. Each datum represents antibody titer in each mouse and the black horizontal lines represent geometric mean for each group. B) The mice in (A) were vaginally challenged with $\sim 3 \times 10^6$ IU of HPV PsV16. C) Additional groups of mice were immunized by buccal injection with 100 μg of mixed MS2-L2 VLPs, control MS2 VLPs, and 5 μg of Gardasil-9 vaccine (immunized intramuscularly). The mice were vaginally challenged with $1.6–4.4 \times 10^6$ IU of PsVs 53, 56, and 11. The average radiance (p/s/cm^2/sr) of luciferase expression was determined using Living Image 4.5.5 software. Each datum represents the average radiance of an individual mouse and the lines represent the geometric mean for each group. The P-values for ELISAs and infection assays were determined by unpaired two-tailed *t*-test and unpaired one-tailed *t*-test, respectively.

(SFD without adjuvants; Fig. 5A) were stored at room temperature and 4 °C for 60 days and their integrities were assessed using TEM. As shown in Fig. 5B and C, SFD VLPs stored at either temperature did not disintegrate during storage; their integrities look similar to freshly prepared non-SFD VLPs (Fig. 5D). Moreover, buccal immunization with the SFD mixed MS2-L2 VLPs (stored at room temperature for 60 days) protected mice from oral infection with HPV PsV16 (Fig. 6).

4. Discussion

HPV-associated HNCs are on the rise especially oropharyngeal squamous cell carcinomas (OPSCC), a type of HPV + HNC. The number of HPV-associated OPSSCs has increased by more than 225% within the last half of the century due to an increase in the number of people engaged in oral sex (a route of transmission) (Chaturvedi et al., 2013; Chaturvedi et al., 2011; D'Souza et al., 2009; D'Souza et al., 2016). It has been suggested that if the trend continues, the number of HPV + OPSCCs will surpass those of cervical cancer cases in the United States (Chaturvedi et al., 2011). While there are validated methods (Pap smears) to screen for cervical cancer, there are no validated methods to screen for HNCs, thus making early preventive interventions for HNCs very challenging. Preventive measures therefore have to rely on preventing HPV infection of the head and neck region during oral sex.

Two prophylactic vaccines (Gardasil-9 and Cervarix; Gardasil-4 is discontinued) are currently approved to protect against HPV infection (FDA, 2018a, 2018b; Zhai and Tumban, 2016). Gardasil-4, which protected against HPV6, HPV11, HPV16 and HPV18 has been shown to elicit oral IgG antibodies (Parker et al., 2018; Pinto et al., 2016); oral

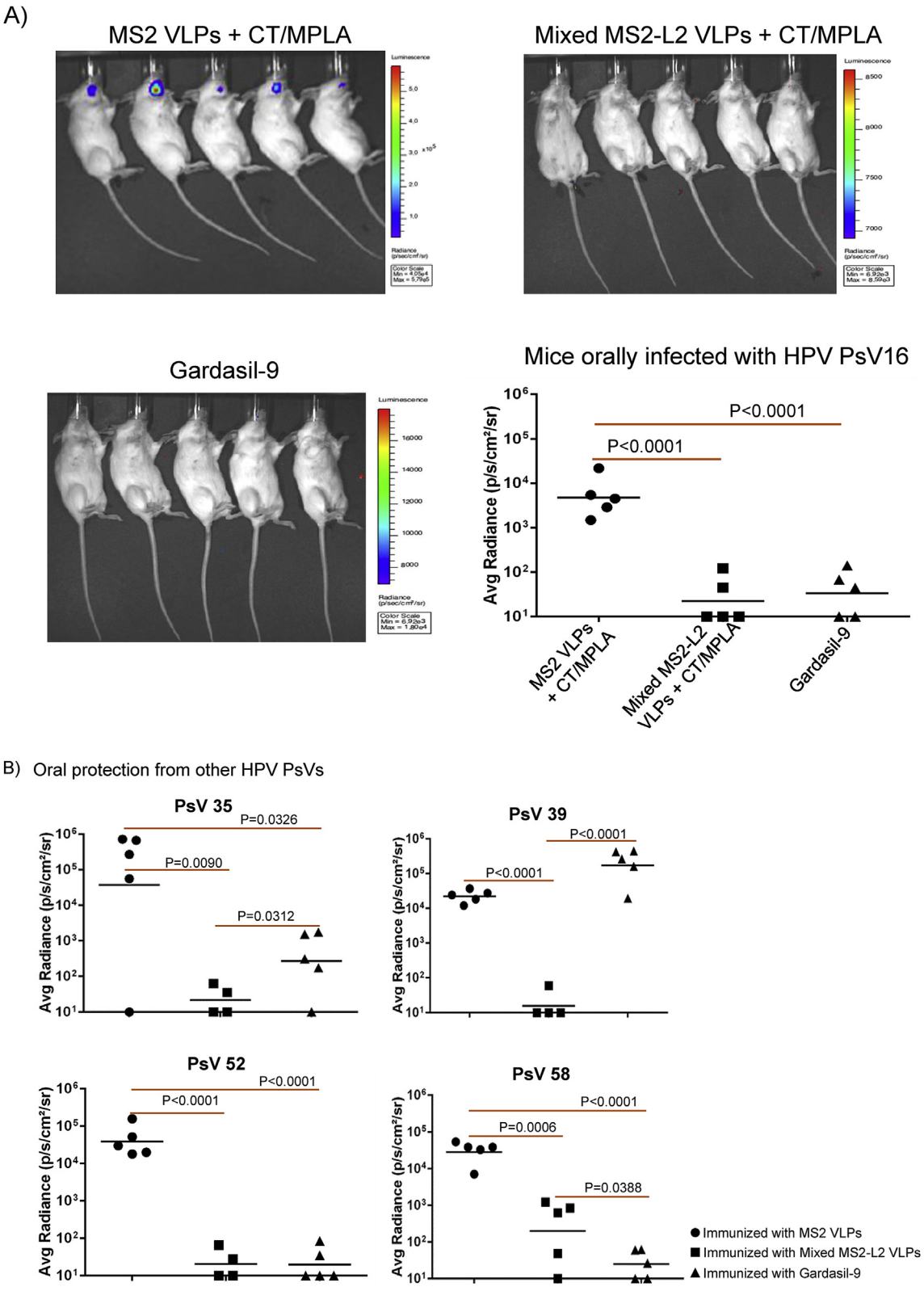

Fig. 2. Buccal immunization with mixed MS2-L2 VLPs and oral protection from HPV PsVs 16, 35, 39, 52 and 58. Mice immunized (buccal injection) thrice (at two-week intervals) with 100 μg of mixed MS2-L2 VLPs, control MS2 VLPs, and 5 μg of Gardasil-9 vaccine (immunized intramuscularly) were orally challenged with: A) 7×10^6 IU of PsV16 and B) 1–7×10^6 IU of PsVs 35, 39, 52 and 58. Each datum represents the average radiance of an individual mouse and the lines represent the geometric mean for each group. The P-values were determined by unpaired one-tailed *t*-test.

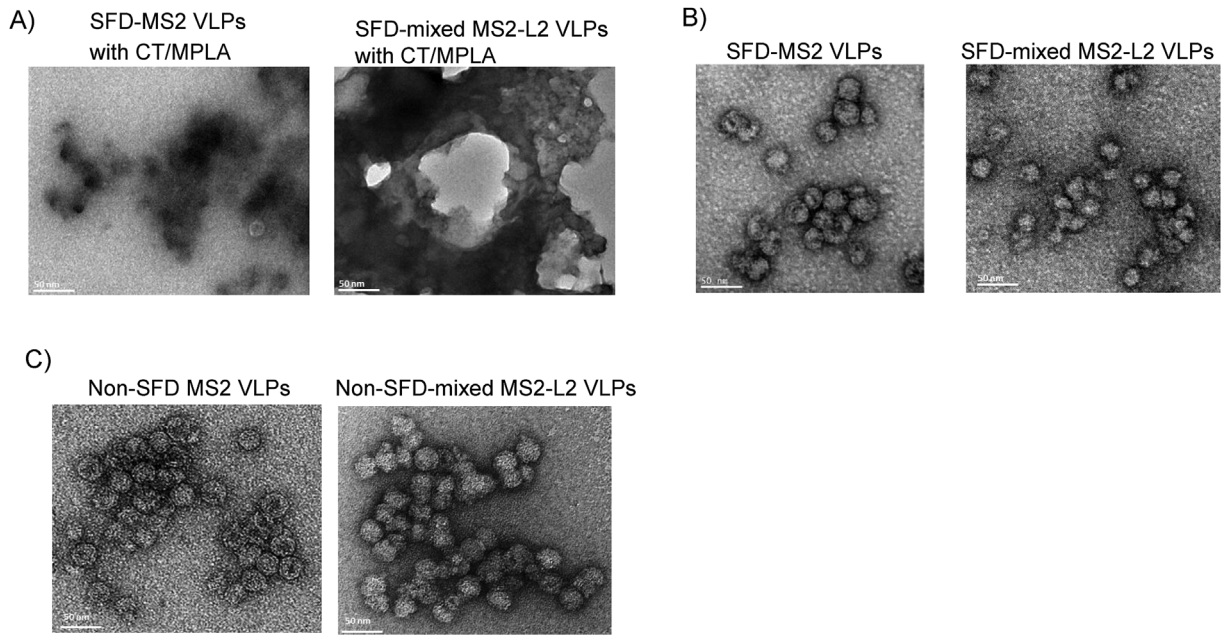

Fig. 3. Transmission electron microscopy (TEM) images of spray-freeze dried (SFD) VLPs and non-spray-freeze dried VLPs. Mixed MS2-L2 VLPs and MS2 control VLPs were SFD with/without mucosal adjuvants (CT and MPLA) and the dry powders were reconstituted in PBS and analyzed (in comparison with fresh liquid VLPs) using a TEM. A) TEM of spray-freeze dried VLPs with CT/MPLA, B) TEM of spray-freeze dried VLPs without CT/MPLA, and C) TEM of fresh liquid VLPs. Images were taken under 30,000X magnification.

antibodies neutralize/protect against HPV6, HPV16, HPV18 (Ahn et al., 2018; Handisurya et al., 2016), and reduce oral prevalence of vaccine types (HPV types included in the vaccine) in vaccinees (Chaturvedi et al., 2018; Fortes et al., 2017; Hirth et al., 2017). However, a protective effect of the vaccine on HPV types not included in the vaccine was not observed in the same studies. The second-generation HPV vaccine, Gardasil-9, has VLPs from seven oncogenic HPV types (HPV16, 18, 31, 33, 45, 52, 58) and two non-oncogenic HPV types (HPV6, 11) included in the vaccine (Cuzick, 2015; Zhai and Tumban, 2016; Zhang et al., 2017). Although no studies have assessed the efficacy of the vaccine against oral HPV infection, like Gardasil-4, it is anticipated that the vaccine will offer oral protection against HPV types included in the vaccine; Gardasil-9 is therefore expected to protect against ~93% HPV + HNCs and > 90% recurrent respiratory papillomatosis, respectively, based on the implication of each HPV type in HNCs (Castellsague et al., 2016; Kreimer et al., 2005; Zhai and Tumban, 2016). As an alternative to Gardasil-9, we developed a bacteriophage-based MS2-L2 VLP candidate vaccine against HPVs (Zhai et al., 2017). Mixed MS2-L2 VLPs [a mixture of MS2-31L2/16L2 and MS2-consL2(69–86)] neutralized and offered vaginal protection against six oncogenic HPV pseudovirus types (HPV16, 18, 31, 33, 45 and HPV58 associated with ~87% of cervical cancer) following intramuscular immunization. It has been reported that mucosal vaccines compared to injectable vaccines provide additional secretory antibody protection against pathogens at mucosal site (Rhee et al., 2012). Here, we assessed the immunogenicity of mixed MS2-L2 VLPs following oral immunization in the presence of two mucosal adjuvant combinations, cholera toxin and MPLA, that target different immune response pathways. Cholera toxin is a mucosal adjuvant that binds to ganglioside receptors (on all cells including epithelial cells, dendritic cells, macrophages, B- and T-lymphocytes) (Bharati and Ganguly, 2011; Cox et al., 2006; Freytag and Clements, 2005) and activates the mitogen activated protein kinase signal transduction pathway thus activating the immune system. MPLA, an approved vaccine adjuvant, binds to toll-like receptors (TLR4 on B cells, dendritic cells, and macrophages) and activates the MyD88 signaling pathway, which also activates the immune system (Cox et al., 2006; Freytag and Clements, 2005). Our results show that buccal immunization of mixed MS2-L2 VLPs without adjuvant offers only suboptimal protection from vaginal infection (Fig. 1B). However, in the presence of one of the two mucosal adjuvants (CT and MPLA), the protection level was enhanced; moreover, robust protection was observed when the two-adjuvant combinations were used in immunization (Fig. 1Band C); these results are consistent with previous studies, which showed that the immunogenicity of an antigen, as well as the longevity of response, can be enhanced by immunizing with more than one adjuvant combination (Ma et al., 2012; O'Neill et al., 2002; Song et al., 2008; Toubaji et al., 2007; Zhang et al., 2011). These results suggest that targeting different immune signaling pathways, simultaneously, is a better strategy to enhance mucosal immune responses at the genital and the oral regions. This was further demonstrated by the level of protection observed at the oral region. Mixed MS2-L2 VLPs protected against five HPV types (HPV16, 35, 39, 52, 58) associated with ~80% of HNCs (Fig. 2A and B). The protection levels against HPV PsVs 35 & 39 were superior to that offered by Gardasil-9 vaccine (which does not include HPV35 & 39 virus-like particles). Given the fact that HPV6 is associated with 64–66.6% of recurrent respiratory papillomatosis (Kocjan et al., 2013; Omland et al., 2014), we also assessed whether mixed MS2-L2 VLPs can protect against oral HPV infection with HPV PsV6. Unfortunately, control mice immunized with MS2 VLPs and infected with HPV PsV6 did not show any visible heat map signal at the oral region even though the IVIS software detected a high infectivity signal at the oral region in control mice compared to mice immunized with the mixed MS2-L2 VLPs (data not shown). To the best of our knowledge, this is the first study that has assessed oral protection using diverse HPV pseudovirus types and has assessed, simultaneously, protection at the oral & the genital regions in the same mice. For example, the groups of mice infected orally with PsV35 (Fig. 2B) were simultaneously infected vaginally with PsV56 (Fig. 1C).

In a previous study, we observed that an MS2 VLP, MS2-16L2, with an HPV L2 insertion (amino acid 17–31 from HPV16) disintegrated when stored at room temperature for 1 month (Tumban et al., 2015); this made the MS2-16L2 VLPs less suitable for developing countries with poorly developed refrigeration & temperature-monitoring infrastructures for transportation and storage of vaccines. To this end, we

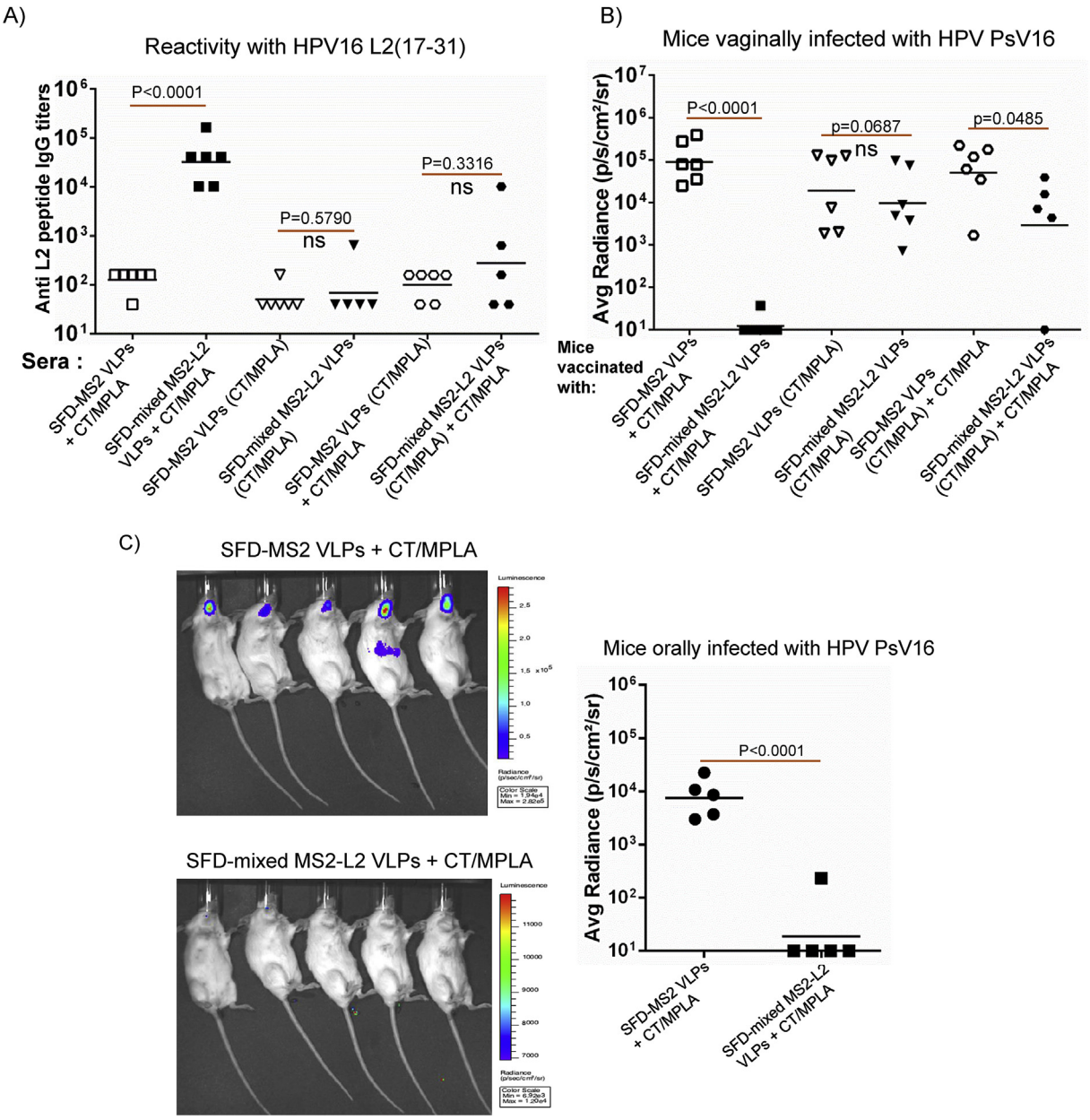

Fig. 4. Buccal immunization with reconstituted SFD mixed MS2-L2 VLPs, vaginal and oral protection from HPV PsV16. Spray-freeze dried VLPs were reconstituted in PBS and mice were immunized thrice with SFD VLPs with/out addition of fresh CT/MPLA adjuvants. A) Sera were collected two weeks after the last immunization and anti-L2 peptide IgG titers in sera were determined by end-point dilution ELISA using 16L2 (17–31) peptide. Each datum represents antibody titer in each mouse and the black horizontal lines represent geometric mean for each group. B) The mice in (A) were vaginally challenged with $\sim 3 \times 10^6$ IU of HPV PsV16. C) Mice immunized (buccal injection) with reconstituted SFD mixed MS2-L2 VLPs or control MS2 VLPs (in the presence of fresh CT/MPLA adjuvants) were orally challenged with $\sim 7 \times 10^6$ IU of PsV16. Each datum represents average radiance of an individual mouse and the lines represent the geometric mean for each group. The P-values for ELISAs and infection assays were determined by unpaired two-tailed *t*-test and unpaired one-tailed *t*-test, respectively.

assessed the potential of formulating mixed MS2-L2 VLPs together with CT and MPLA into a dry powder, with the ultimate goal of enhancing the thermostability of the candidate vaccine. We SFD the VLPs together with the mucosal adjuvants. However, the VLPs agglomerated when SFD with the adjuvants and were not immunogenic even with the addition of more adjuvant prior to immunization (Fig. 4A). Given this setback, we tried another technique, spray drying, to formulate the VLPs into dry powder; however, similar results (agglomeration) were obtained (data not shown). These results are consistent with another study, which showed that spray drying of vaccines with adjuvant (alum) leads to vaccine agglomeration (Maa et al., 2003). Taken together, these results suggest that certain antigens may not be amenable to SFD or spray drying with adjuvants. Irrespective of this, the mixed MS2-L2 VLPs were successfully SFD into dry powder without the adjuvants and could be stored at room temperature, without the VLPs disintegrating, for up to 60 days (Figs. 3 and 5). Moreover, the SFD VLPs offered robust protection from oral HPV infection.

In summary, the display of a concatemer of epitope 17–31 from HPV16L2 & epitope 20–31 from HPV31 on one VLP and the display of consensus epitope 69–86 on another VLP (Zhai et al., 2017) is an excellent approach to elicit broad protection against diverse HPV types. Mixed MS2-L2 VLPs [MS2-31L2/16L2 VLPs and MS2-consL2(69–86)] is a candidate HPV vaccine with the potential to offer protection against multiple HPV types at the oral and genital regions. In a previous study (Zhai et al., 2017), we showed that the candidate vaccine offers protection from genital infection against six oncogenic HPV types (HPV16,

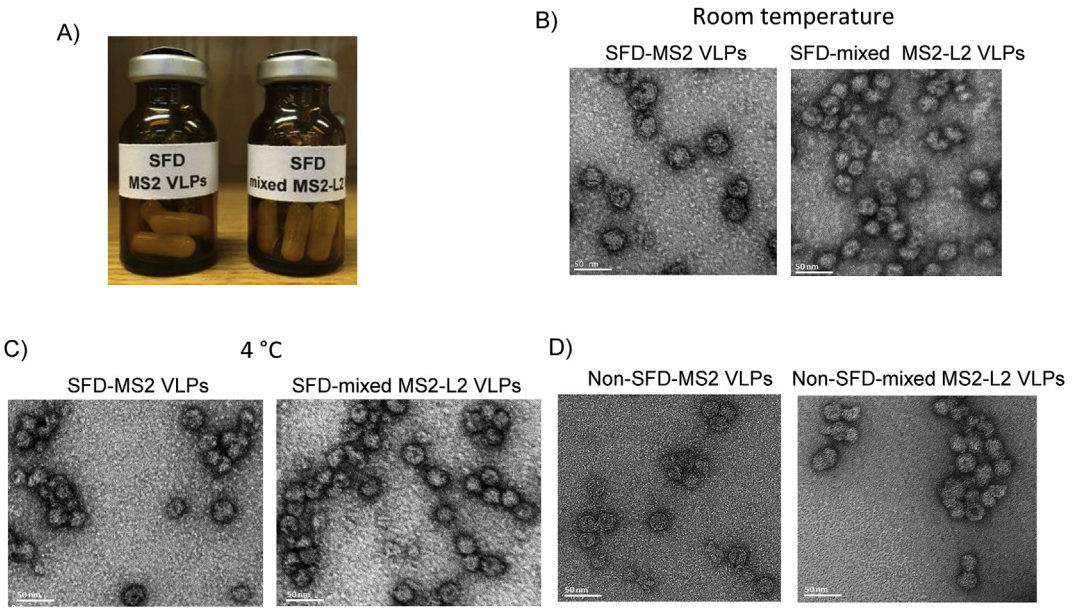

Fig. 5. TEM images of SFD VLPs after two months storage. Mixed MS2-L2 VLPs and MS2 control VLPs spray-freeze dried without adjuvants were loaded into capsules, sealed in amber-colored Wheaton glass bottles, and purged with nitrogen gas (A). The vials with the VLPs were stored at room temperature or 4 °C for 60 days. After 60 days, the spray-freeze dried VLPs were removed from the capsules, reconstituted in PBS buffer. TEM was done and images of the VLPs stored at: B) room temperature and C) 4 °C taken. The VLPs were compared to non-SFD VLPs (D). Images were taken at 30,000X magnification.

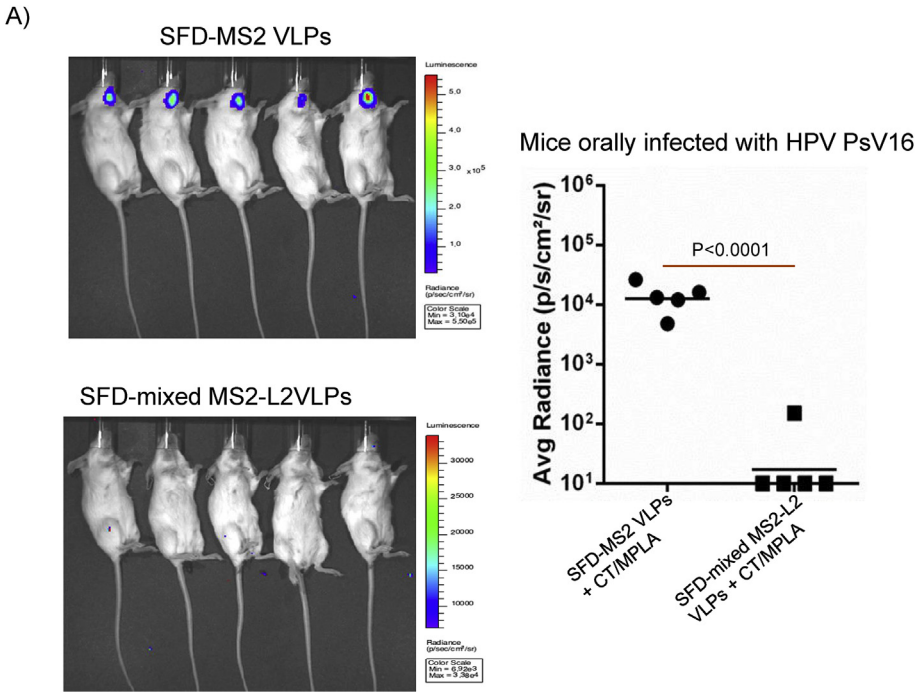

Fig. 6. Buccal immunization with reconstituted SFD mixed MS2-L2 VLPs after 60-day storage and oral protection from HPV PsV16. Spray-freeze dried VLPs were reconstituted in PBS after 60-day storage at room temperature. The reconstituted VLPs in the presence of fresh CT/MPLA adjuvants were used to immunize mice thrice. Immunized mice were orally challenged with 7×10^6 IU of HPV PsV16. Each datum represents the average radiance of an individual mouse and the lines represent the geometric mean for each group. The P-values were determined by unpaired one-tailed *t*-test.

18, 31, 33, 45, and 58). In the current study, we show that the candidate vaccine offers protection from genital with HPV PsVs 11, 53, 56 and protection from oral infection with HPV PsVs 16, 35, 39, 52, 58. Although we did not assess, in the current study, protection from oral infection with HPV PsVs 18, 31, 33, 45, 53, 56 and PsV11, we expect immunized mice to offer oral protection against these viruses given the level of protection that was observed at the vaginal region with these PsVs in our previous (Zhai et al., 2017). This view is supported by the fact that mice immunized with the mixed MS2-L2 VLPs, in our previous study (Zhai et al., 2017), protected the mice from vaginal infection with HPV PsV16 (at levels similar to Gardasil-9) and similar levels of protection against PsV16 were observed at the oral region in the current study. Overall, mixed MS2-L2 VLPs has the potential to orally protect against eleven oncogenic HPV types (16, 18, 31, 33, 35, 39, 45, 52, 53, 56, and 58) associated with ~99% of HNCs (estimates are based on the contribution of each HPV type to HNCs (Castellsague et al., 2016; de Martel et al., 2017; Kreimer et al., 2005; Ndiaye et al., 2014)). The candidate vaccine also has the potential to protect, genitally, against the same eleven oncogenic HPV types associated with ~95% of cervical cancer (estimates are based on the contribution of each HPV type to cervical cancer (de Sanjose et al., 2010; Zhai and Tumban, 2016)). The candidate vaccine also shows protection against HPV11 associated with 19–32% of recurrent respiratory papillomatosis (Kocjan et al., 2013; Omland et al., 2014) and 36% of genital warts (Chang et al., 2013;

Sturegard et al., 2013). Thus, mixed MS2-L2 VLPs is a next generation HPV vaccine that should be evaluated further, especially for patients infected with HIV or are suffering from AIDS. HPV type distribution in HIV patients seems to be different from the normal population (Zhai and Tumban, 2016); in addition to the other HPV types (16, 18, 31, 33, 45, 52, 58) that are common in the normal population, HIV patients are infected or co-infected mostly with HPV35, 39, 53, 56, which are all protected by mixed MS2-L2 VLPs.

Financial interest and conflict of interest disclosures

Ebenezer Tumban is a co-inventor of L2 bacteriophage virus-like particles-related patent applications licensed to Agilvax Biotech by the University of New Mexico. Interactions with Agilvax Biotech are managed by the University of New Mexico in accordance with its conflict of interest policies.

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