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A broadly protective thermostable next generation HPV vaccine based on a concatemer peptide and a consensus peptide of L2 displayed on bacteriophage virus-like particles

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**A BROADLY PROTECTIVE THERMOSTABLE NEXT
GENERATION HPV VACCINE BASED ON A
CONCATEMER PEPTIDE AND A CONSENSUS PEPTIDE
OF L2 DISPLAYED ON BACTERIOPHAGE VIRUS-LIKE
PARTICLES**

By

Lukai Zhai

A DISSERTATION

Submitted in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

In Biological Sciences

MICHIGAN TECHNOLOGICAL UNIVERSITY

2019

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This dissertation has been approved in partial fulfillment of the requirements for the Degree of DOCTOR OF PHILOSOPHY in Biological Sciences.

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Preface

The *In vitro* neutralizing assays in Chapter 1 were conducted by Susana Pang in Dr. John Schiller's lab (Laboratory of Cellular Oncology, National Cancer Institute, National Institute of Health). Sera used in this experiment were collected in Michigan Technological University and were sent to our collaborator for further studies. The HPV pseudovirus challenge experiments in Chapter 1 were conducted by Julianne Peabody in Dr. Bryce Chackerian's lab (Department of Molecular Genetics and Microbiology, University of New Mexico School of Medicine). The data were published in "Antiviral Research 147 (2017) 116-123". The Spray-freeze drying formulation into dry powder was conducted by Dr. Nitesh Kunda in Dr. Pavan Muttill's lab (Department of Pharmaceutical Sciences, College of Pharmacy, University of New Mexico). We sent the liquid VLPs samples to them and they sent us the lyophilized VLPs samples. Data in Chapter 2 has been published in the journal of Antiviral Research (2019) (<https://doi.org/10.1016/j.antiviral.2019.03.012>).

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Abstract

Current human papillomavirus (HPV) vaccines (Cervarix and Gardasil-9) are highly immunogenic and derived from the major capsid proteins (L1) of different HPV types. L1 is not conserved among different HPV types. Thus, these L1-based vaccines protect mostly against the HPV types included in the vaccines with minimal cross-protection against non-vaccine HPV types. In addition to this, the vaccines require refrigeration during transportation and storage. To broaden protection against diverse HPV infection, we targeted epitopes (17-31, 69-86, and 108-122) on the major capsid protein (L2) of HPV, which are conserved among different HPV types for vaccine design. We explored the display of these L2 epitopes, individually or as concatemers, on the surface of a highly immunogenic bacteriophage MS2 virus-like particle (VLP) platform. VLPs are empty viral shells without a viral genome. They are derived from viral structural proteins such as envelope proteins or capsid proteins. VLPs cannot replicate, they are non-infectious, and are highly immunogenic; thus, they are safe and effective platforms for vaccine designs. Mice immunized with the MS2-L2 candidate HPV vaccine (a mixture of MS2 VLPs: one displaying a concatemer of peptides from HPV16L2 & HPV31L2 and the other displaying a consensus peptide, epitope 69-86, from the alignment of 23 HPV types) elicited high-titer antibodies that protected mice from genital infection with nine diverse HPV types; the VLPs also protected mice from oral infection with five diverse HPV types. To

improve the thermostability of the MS2-L2 VLPs, the candidate vaccine was formulated into powder by spray-freeze drying technique. Spray-freeze dried VLPs stored at room temperature for two months were still immunogenic and offered protection against HPV infection. Mixed MS2-L2 VLPs is a candidate next generation HPV vaccine. We also explored, in this dissertation, the potential of developing a novel thermostable VLP platform based on a thermophilic bacteriophage, Φ IN93. Our results seem to suggest that co-expression of two truncated versions of coat proteins from Φ IN93 have potential to form structures that resemble VLPs.

Background

Immunity, vaccination and virus-like particles (VLPs):

The human body is well protected from infectious pathogens such as bacteria and viruses by the complex immune system. The immune system is divided into the innate immune system (innate immunity) and the adaptive immune system (adaptive immunity). Innate immunity provides the first line of defense against general pathogens. It does not show specificity to a particular antigen (antigen-independent), its response is immediate (from minutes to hours) after foreign invasion, and it does not have memory responses. Three components provide innate immunity: physical (skin) & chemical barriers (mucus, tears and saliva), phagocytes (blood monocytes, neutrophils and macrophages), and pattern recognition molecules [1]. When innate immunity fails to clear off invasions, adaptive immunity is activated. Adaptive immunity has different features compared to innate immunity: i) it takes longer time (several days) for the response to be mounted; ii) it is more specific to the antigens (antigen-dependent); iii) it generates memory to the invaded pathogens, which means adaptive immunity can rapidly recognize and response to the same antigens following reinfection (with the same pathogen) with the help of the existing memory cells or antibodies [2]. Adaptive immunity contains more complex components such as T and B lymphocytes (T cells and B cells) and antibodies. Antibody response or humoral immunity is one of the two main responses of

adaptive immunity (antibodies are produced by B cells). The other response, cell-mediated immunity is mounted by T cells. As shown in Figure 1, the generation and secretion of antibodies involves collaboration of a series of immune cells: antigen-presenting cells (APCs) such as macrophages, B cells, and dendritic cells ingest pathogens, digest the pathogens, and present antigens from the pathogen, on cell surfaces in association with the major histocompatibility complex (MHC) class II molecules. CD4 T cells interact with APCs, which displayed the antigens in association with MHC II. CD4 T cells are then activated and they release cytokines such as IL-4, IL-5 and IL-6. The released cytokines or the cross-linking of B cell receptors can lead to B cells activation and differentiation into plasma B cells (secrete antibodies) and memory B cells which provide a rapid and long-lasting response on reinfection with the same pathogen [3, 4]. Innate and adaptive immunities do not work independently, but rather are complementary in maintaining host defense. While some pathogens can be eliminated by the immune system, some will develop ways to escape from host defense network to cause deadly diseases or cancers after infection. Thus, vaccination became an important strategy to prevent some diseases and cancers.

Fig. 1. Generation and secretion of antibodies through T cell and B cell activation. Antigen invasion (green) can trigger phagocytosis whereby antigens are phagocytosed and digested by antigen presenting cells (APCs). APCs then display the processed antigens on surface together with MHC II molecules. Antigens on MHC II are presented to receptors on CD4 T cells causing CD4 T cells activation. The activated CD4 T cells release cytokines to further activate naïve B cells as well as the CD4 T cells. Activated B cells differentiate into antibody secreting plasma cells (release antibodies against the antigen) and

memory B cells (for a rapid and effective response against reinfection with the same antigen). In addition to this pathway, the antigens also can directly bind to B cells by cross-linking with B cell receptors, leading to B cell activation. The activated B cells differentiate into plasma cells. This type of B cell activation is associated with little or no memory to the response.

Vaccination is defined as the administration of an antigen to the body to enable the body to develop an immune response with the ultimate goal of preventing the body from being infected by the infectious agent from which the antigen was derived. In other words, a vaccine is an imitation of real pathogen (organism) or they are antigens derived from a pathogen but without the potential to infect or cause a disease. The organism can be live but attenuated, it can be inactivated; alternatively, the vaccine can be an antigen, which is a subunit or part of a real pathogen or it could be a toxoid (non-toxic form of a toxin) derived from pathogens; either of them can activate the host adaptive immune response to offer protection against the real pathogen invasion [5, 6]. Adjuvants are often used as part of a vaccine to enhance activation of the innate immune system and thus protection. Diverse classes of compounds can be used as adjuvants including aluminum salts, emulsions, saponins, liposomes and micro-particles [7]. Adjuvants can enhance the immune responses through diverse mechanisms such as formation of a depot at the injection site for slow and constant stimulation, up-regulation of cytokines & chemokines for recruitment of innate

immune cells, increase in antigen presentation to APCs, and activation and maturation of APCs [8]. Safety and high immunogenicity are considered as two important factors for developing a new vaccine. For example, attenuated vaccines are highly immunogenic because the viruses are still alive and usually one or two doses can achieve long-lasting protection; however, for people who have weakened immune system or transplanted organs, live viruses might cause diseases in vaccinees. The inactivated vaccines are safe for patients but need more boosts to achieve and maintain a high level immunogenicity. Among all these types of vaccines, multi-protein structured vaccines, based on virus-like particles (VLPs) seem to be attracting a lot of attention as an approach in developing next-generation preventive vaccines against infections agents [9].

VLPs are empty viral shells without viral genome formed by viral structural proteins such as envelope proteins or capsid proteins. These structural proteins can spontaneously assemble to form VLPs without the viral genomes; VLPs therefore cannot replicate, they are non-infectious, and thus, they are safe for vaccine designs. They are highly immunogenic; the sizes and the icosahedral structures make them easy for APCs to recognize and take them up for processing and antigen presentation to CD4 T cells. Also, repetitive copies of antigens displayed on VLPs enable them to trigger strong immune responses especially the cross-linking of B cell receptors on the surface of B cells. VLPs have been used to develop vaccines against human papillomaviruses (HPVs) [10, 11] and hepatitis B virus (HBV) [12]. VLPs derived from HPV are highly

immunogenic and protect against HPV-associated cancers (described in detail below).

HPV, HPV vaccines, and limitations:

HPVs are non-enveloped DNA viruses, which infect human stratified epithelial cells. More than 200 HPV types have been identified and among them, 42 types are transmitted sexually [13, 14]. HPVs are the most common sexually transmitted infections [15]. Ninety percent of HPV infections are cleared within 24 months after infection [16]. However, persistent infection with mucosal types can lead to neoplasias [17]. HPV can be divided into two groups based on their association with neoplasias: low-risk types and high-risk types. Infections with the low-risk types (such as types 6, 11, 40, 42, 43, 44, 54, 61, 72, 81) are associated with genital warts as well as recurrent respiratory papillomatosis (RRP). HPV types 6 and 11 alone are associated with about 90% of genital warts and about 95% of RRP [18-21]. On the other hand, infections with the high-risk types (such as 16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 70, 73, 82) are associated with cancers such as cervical [18, 22], penile [23, 24], vulvar, anal, and vaginal [25-27] cancers as well as head and neck squamous cell carcinomas (including oral, oropharyngeal, and laryngeal cancers) [28].

Currently, two prophylactic vaccines, Cervarix (bivalent HPV), and Gardasil-9 (nonavalent HPV), have been approved by the Food and Drug Administration (FDA) in the United States to protect against HPV infections [29]. These vaccines

are composed of virus-like particles (VLPs) derived from the major capsid protein (L1) of HPV. The HPV vaccines are highly immunogenic. For example, as shown in Figure 2, the Gardasil 9 protects against ~90%, worldwide average, of cervical cancer cases caused by HPV16, 18, 31, 33, 45, 52, 58 and it also protects against ~90% of genital warts cases caused by HPV6 and 11 [30]. Gardasil-9 is also expected to protect against ~80-85% cases of HPV-associated vaginal cancers, 90-95% of HPV-associated anal cancers, 85-90% of HPV-associated vulvar cancers [30], and ~86% of penile cancers [24]. However, the current vaccines still have some limitations.

Fig. 2. Worldwide estimated percentages of HPV-associated cancers, HPV-associated warts, and papillomatosis to be protected by Gardasil-9.

Estimates are based on the prevalence and the sum of percent contributions of vaccine HPV types to different HPV-associated (cervical, vaginal, anal, vulvar, penile, head and neck) cancers, genital warts including recurrent respiratory papillomatosis. For example, 90% protection against cervical cancer is based on the fact that HPV16 is associated with (~55.4%), HPV18 (16.1%), HPV45 (4.7%), HPV31 (3.8%), HPV33 (4.1%), HPV52 (2.8%), and HPV58 (3.0%) of cervical cancer cases worldwide.

First, they offer protection mostly against HPV types included in the vaccines but with less or no protections against non-vaccine HPVs. This is because they are derived from L1 proteins, which are not conserved among HPV types; they protect mostly against the HPV types included in the vaccines with little cross-protection against non-vaccine HPV types (e.g. HPV31, 45, 58) [31-35]. People who are infected with HPV, especially HIV patients, are able to be infected with multiple HPV types: up to 10 HPVs were found in one HIV-infected patient [36]. Thus, an optimal HPV vaccine should protect against more cancer-causing HPVs to decrease the risk of developing HPV-associated cancers. Secondly, since little is known about the ability of current vaccines to protect against oral HPV infections, it is important and necessary to go further to study the efficacy of the vaccine against diverse oral HPVs infections. Thirdly, the current vaccines are thermosensitive; thus, refrigeration and temperature monitoring facilities are required for the vaccines. Refrigeration equipment is not available worldwide

especially in the developing countries and regions, where there are higher rates of HIV infections.

Fig. 3. HPV genome and capsid structure model. HPV genome is circular, but it is shown in the linear form (top image) for simplicity. Genes coding for early proteins (E1, E2, E4, E5, E6 and E7), which are non-structural proteins are shown in green. Genes coding for late proteins are L1 and L2; L1 (shown in blue) forms pentamers and each pentamer has one L2 (shown in red) at the vertex of

each pentamer. The icosahedral capsid (bottom; surrounds the genome) is composed of major capsid protein (L1) and minor capsid protein (L2).

With this in consideration, other studies have been looking into designing a next generation HPV vaccine, targeting the minor capsid protein, L2, shown in Figure 3. The minor capsid protein, especially the N-terminus, is highly conserved among diverse HPV types [37, 38]. Nevertheless, normal infection does not induce protective anti-L2 antibody responses because L2 is only transiently exposed on the capsid. During HPV infection, the capsid binds to heparan sulfate proteoglycan (HSPG) on the basement membrane; the capsid then undergoes a series of conformational changes thus transiently exposing the N-terminal portion of L2 [39, 40]. This conformational change allows the virus to bind to and infect epithelial cells. If neutralizing antibodies, following immunization with L2 antigens are available, they can bind to exposed-neutralizing L2 epitopes thus preventing the infection [39, 40] (Fig. 4). Neutralizing L2 antibodies can cross-protect against diverse HPV types [41-43]. The L2 protein, unlike the L1 protein, cannot form VLPs and thus, it is less immunogenic. Thus, different approaches have been explored to enhance the immunogenicity of L2 protein; these include conjugation of an L2 peptide to thioredoxin [44, 45], construction of L2 concatemer proteins derived from different HPV types [46, 47], and the display of L2 peptides on different types of VLPs [48-52]. While these approaches enhance cross-protection against diverse HPV types, they do not offer complete protection

against all cancer-causing HPV types. VLPs derived from viruses that infect bacteria (bacteriophages e.g. MS2, PP7 and QB) have also been used as platforms to multivalently display less immunogenic peptides from other pathogens in order to increase the immunogenicity of the peptides [49, 53]. In this dissertation, I focused on developing a candidate L2 vaccine against HPV based on a bacteriophage MS2 VLP platform. In addition to this, I explored the potential to develop a novel thermostable VLP platform based on a virus, Φ IN93, which infects a thermophilic bacterium.

Fig. 4. Schematic (adapted from [39]) illustrating HPV infection and how L2 antibodies targeting L2 protein prevents HPV infection. Micro-trauma or a cut allows HPVs to: 1) bind to heparan sulfate proteoglycan (HSPG) on basement membrane cells. 2) After conformational change, pre-buried L2 will be shortly exposed and cleaved for another conformational change, which allows the viruses to 3) recognize receptors (integrin proteins) on epithelial cells and

infection of the cells are initiated. 4) In the presence of anti-L2 antibodies, binding to the receptor is blocked and infection is prevented.

Special aims

The goals of my dissertation were to develop a candidate prophylactic HPV vaccine, which can protect against more high-risk HPVs infections (including protection from oral HPV infections) and to formulate the candidate vaccine into a thermostable product that can be transported and stored without the need to refrigerate the vaccine. In a previous study, an HPV16 L2 epitope containing amino acids (aa) 17-31 displayed on MS2 bacteriophage VLPs offered protection against up to 11 HPV types (HPV5, 6, 16, 31, 33, 35, 39, 45, 51, 53, and 58) [49]. However, there was suboptimal protection against some of these HPVs including those associated with HNCs. To broaden and enhance protection, in this dissertation, we attempted to display more epitopes on the same VLPs already displaying HPV16 L2 epitopes. Additionally, we assessed oral immunity/protection following oral immunization with our L2 phage VLPs together with a combination of different mucosal adjuvants. Furthermore, we used spray-freeze drying technique to convert the liquid VLPs into a thermostable dry powder vaccine. We also assessed whether coat proteins from a thermophilic phage Φ IN93 can assemble into VLPs. Our hypotheses are: i) immunization with multi-epitope L2 VLPs can offer broader protection against more HPV types; ii) a spray-freeze dried VLPs powder vaccine will be more stable for storage at room temperature; iii) coat proteins of Φ IN93, expressed in *E. coli*, can assemble into VLPs.

Aim 1) Develop and test a candidate vaccine with multiple epitopes displayed on MS2 bacteriophage VLPs. Sequences of all high-risk HPVs and some wart-causing low-risk HPVs were aligned and two consensus epitopes that have the potential to broaden protection against diverse HPVs were chosen and used to design a candidate HPV vaccine (**Chapter 1**).

Aim 2) Assess the ability to enhance oral immune response using a combination of mucosal adjuvants. Mucosal adjuvants such as cholera toxin (CT) and monophosphoryl lipid A (MPLA) are able to stimulate signaling pathways associated with immune responses and thus they may enhance oral immune responses (**Chapter 2**)

Aim 3) Use the spray-freeze drying technique to formulate L2 vaccine with/without mucosal adjuvants to a thermostable powder. This strategy can avoid the problems of vaccine refrigeration during transportation and storage in developing countries (**last part of Chapter 2**)

Aim 4) Develop a novel VLP platform based on thermophilic bacteriophage Φ IN93 coat proteins (**Chapter 3**)

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Chapter 1

A Novel Candidate MS2 Phage VLP Vaccine Displaying a tandem HPV L2 Peptide offers Similar Protection in Mice to Gardasil-9

1.1 Abstract

Human papillomaviruses (HPVs) cause approximately 5% of cancer cases worldwide. Fortunately, three prophylactic vaccines have been approved to protect against HPV infections. Gardasil-9, the most recent HPV vaccine, is predicted to offer protection against the HPV types that cause ~90% of cervical cancer, 86% of HPV-associated penile cancers, and ~93% of HPV-associated head & neck cancers. As an alternative to Gardasil-9, we developed and tested a novel candidate vaccine targeting conserved epitopes in the HPV minor capsid protein, L2. We displayed a tandem HPV31/16L2 peptide (amino acid 17-31) or consensus peptides from HPV L2 (amino acid 69-86 or 108-122) on the surface of bacteriophage MS2 virus-like particles (VLPs). Mice immunized with the MS2 VLPs displaying the tandem peptide or immunized with a mixture of VLPs (displaying the tandem peptide and consensus peptide 69-86) elicited high titer antibodies against individual L2 epitopes. Moreover, vaccinated mice were protected from cervicovaginal infection with HPV pseudoviruses 16, 18, 31, 33, 45, and 58 at levels similar to mice immunized with Gardasil-9. These results

suggest that immunization with a tandem, L2 peptide or a low valency mixture of L2 peptide-displaying VLPs can provide broad protection against multiple HPV types.

1.2 Introduction

Human papillomaviruses (HPVs) are the most common sexually transmitted infections; approximately 40 HPV types can be transmitted sexually and they cause neoplasias such as genital warts and cancers [1, 2]. Persistent infection with high-risk HPV (HR-HPV) types (HPV16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 70, 73, 82) is associated with ~94% of anal cancers, ~63% of penile cancers, ~74% of vaginal cancers, ~5-20% of head & neck squamous cell carcinomas, and nearly all cases of cervical cancers [3-5]. Infections with low-risk HPV types (HPV6, 11, 43, and 44) are associated with genital warts [6-8]. HPV-associated cancers can be initiated by infection with a single HR-HPV type or by infection with multiple HR-HPV types [9, 10]; a situation that occurs especially in human immunodeficiency virus (HIV) and acquired immunodeficiency syndrome (AIDS) patients. HIV/AIDS patients are more susceptible to HPV-associated cancers compared to non-HIV infected patients [9, 11-15]. Thus, these patients need broad protection from infections by multiple HPV types.

Two prophylactic vaccines (Cervarix, and Gardasil-9) have been approved to protect against certain types of HPV infections. The vaccines are composed of virus-like particles (VLPs) derived from HPV major capsid protein (L1). The vaccines are highly immunogenic. However, because L1 neutralizing epitopes are not highly conserved among HPV types, L1 vaccines protect mostly against the HPV types included in the vaccines, with minimal cross-protection against non-vaccine HPV types [16-20]. For example, Gardasil-9 offers protection against the HR-HPV types (HPV16, 18, 31, 33, 45, 52, 58) that cause ~93% of head and neck cancers, ~90% of cervical cancers, 90-95% of anal cancers and more than 80% of vaginal, penile and vulvar cancers. The vaccine also protects against HPV6 and 11, types that cause 90% of genital warts. [4]. As such, women who are vaccinated with Gardasil-9 vaccine (and also the other HPV vaccines) are still advised to continue screening for other HPV types not included in the vaccine. To broaden protection, researchers have focused on developing next-generation HPV vaccines targeting the HPV minor capsid protein (L2) [21-23]. The minor capsid protein, especially the N-terminus, is highly conserved among diverse HPV types [24, 25]. Nevertheless, normal infection does not induce protective anti-L2 antibody responses because L2 is only transiently exposed on the capsid. During HPV infection, the capsid binds to heparan sulfate proteoglycan (HSPG) on the basement membrane; the capsid then undergoes a series of conformational changes thus transiently exposing the N-terminal portion of L2 [26, 27]. This conformational change allows the virus to bind to and infect epithelial cells. If neutralizing antibodies, following immunization with L2 antigens

are available, they can bind to exposed-neutralizing L2 epitopes thus preventing the infection [26, 27]. Neutralizing L2 antibodies can cross-protect against diverse HPV types [28-30]. However, L2-based immunogens generally elicit much lower antibody titers than L1 VLPs because, unlike L1, L2 cannot self-assemble into VLPs. Different approaches have been explored to enhance the immunogenicity of L2 protein; these include conjugation of an L2 peptide to thioredoxin [21, 31], construction of L2 concatemer proteins derived from different HPV types [22, 32], and the display of L2 peptides on different types of VLPs [33-37]. While these approaches enhance cross-protection against diverse HPV types, they do not offer complete protection against all cancer-causing HPV types. In a previous study, we displayed a conserved epitope (representing amino acid 17-31) from HPV16 L2, on the surface of bacteriophage MS2 VLPs. MS2-16L2 VLPs elicited strong antibody response in mice and offered complete protection against HPV16 pseudovirus (PsV) 16 as well as sub-optimal cross-protection against other HPV PsV types [34]. In another study, we showed that immunization with bacteriophage VLPs displaying a consensus L2 epitope (representing amino acid 65-85 derived from an alignment of different HR-HPV types), enhanced cross-neutralization, albeit at low titers, against diverse HPV PsV types (PsV 16, 31, 18, 45 and 58) compared to immunization with VLPs displaying a similar epitope derived from HPV16 [36]. These results suggested that the immunogenicity as well as the spectrum of protection against diverse HPV types could be enhanced by displaying conserved or consensus L2 epitopes on the surface of bacteriophage VLPs. In this study, we assessed the

spectrum of protection against diverse HPV types following immunization with a mixture of two MS2-L2 VLPs in comparison to immunization with Gardasil-9.

1.3 Materials and methods

1.3.1 Cloning, expression and purification of MS2-L2 VLPs

Polymerase chain reaction (PCR) was used, as previously described [38], to insert different HPV L2 epitopes on the N-terminus of the single-chain dimer of MS2 bacteriophage coat protein. Plasmid pDSP62 (which expresses the single-chain dimer of MS2 coat protein) was used as a PCR template (Fig. 1).

Fig. 1. Schema showing insertion of 16 L2 epitope at N-terminus of MS2 bacteriophage and assembly of VLPs.

To get a consensus L2 sequence, amino acid sequences of all HR-HPVs and some low-risk ones (HPVs 6, 11, 34, 43 and 59) were analyzed and aligned by Jalview software and T-coffee program (Fig. 2). The following HPV L2 peptides (Table 1) representing amino acids (aa) sequences were inserted: a tandem peptide (aa 20-31 from HPV31 L2 & aa 17-31 from HPV16 L2), aa 69-86 from a consensus (cons)L2 sequence, aa 108-122 from a consensus (cons)L2 sequence, and a multivalent epitope representing consL2(108-122)/HPV31L2 (20-31)/HPV16L2(17-31).

These L2 sequences were engineered into forward PCR primers. A reverse primer, E3.2 (5' CGGGCTTTGTTAGCAGCCGG 3'), which anneals downstream of a unique BamHI site in the pDSP62 plasmid was used for all PCR amplifications above. Amplified MS2-L2 PCR fragments were cloned into pDSP62 plasmid using NcoI and BamHI restriction sites and the plasmids were then transformed into C41 *E. coli* bacteria. All constructs were sequenced to confirm insertions. To express recombinant MS2-L2 proteins, transformed C41 cells were grown at 37°C until the cells reached an optical density (OD)₆₀₀ of 0.6. Protein expression was induced with 0.5 mM isopropyl β -D-1-thiogalactopyranoside for 4 hours or overnight. Cell pellets were collected and lysed using 0.2% lysozyme solution. Soluble MS2-L2 VLPs were purified by gel filtration on Sepharose CL-4B column.

Table 1: HPV L2 epitopes inserted on MS2 VLPs

Epitope Name	Epitope Sequence	VLP Model
16L2 (aa 17-31)	QLYKTCKQAGTCPPD
31L2 (aa 17-31)	QLYQTCKAAGTCPSD
31L2 (aa 20-31)/16L2 (aa 17-31)	QTCKAAGTCPSDQLYKTCKQAGTCPPD
Consensus L2(aa 69-86)	RTGYVPLGTRPPTVVDVT
Consensus L2(aa 108-122)	LVEESSVIISGAPPP
Consensus L2(aa 108-122)/ 31L2 (aa 20-31)/16L2 (aa 17-31)	LVEESSVIISGAPPPQTCKAAGTCPSDQL YKTCKQAGTCPPD

1.3.2 Characterization of MS2-L2 VLPs

Transmission electron microscopy (TEM) was used to assess whether all constructs assembled into VLPs in a way as previously described [38]. To examine whether the L2 epitopes were displayed on the MS2 bacteriophage VLPs, enzyme-linked immunosorbent assay (ELISA) and Western blot were performed. Western blotting was conducted as follows: 100 ng of MS2-L2 VLPs or control MS2 VLPs were run on an SDS-PAGE gel and transferred to polyvinylidene fluoride membranes. The membranes were blocked and 1:5,000 dilution of anti-HPV16 L2 (aa 1-88) serum [34] or MS2 serum was added and incubated for 2 hours. The membranes were washed and 1:10,000 dilution of

horseradish peroxide (HRP)-conjugated goat anti-mouse IgG antibodies added for 1 hour. The membranes were then washed and developed using a mixture of SuperSignal West Pico (Lumino/Enhance and Stable Peroxide) solutions. To confirm the Western blot results, ELISA was conducted as follows: Briefly, ELISA plates were coated with 500 ng of purified MS2-L2 VLPs. The wells were blocked and serial dilutions of anti-HPV16 L2 (aa 1-88) serum was added. The plates were incubated for 2 hours and 1:5,000 dilution of HRP-conjugated goat anti-mouse IgG antibodies was added for 1 hour. The plates were developed by adding 3, 3', 5, 5'-tetramethylbenzidine (TMB) and the reactivity was determined at OD₄₅₀.

1.3.3 Immunization of mice and characterization of antibody responses

All animal work was conducted in accordance with Institutional Animal Care and Use Committee (IACUC) guidelines. Four Balb/c mice (per group) were immunized intramuscularly (i.m.) with 5µg each of MS2-16L2 VLPs (displaying aa 17-31 from HPV16) [34], MS2-31L2 VLPs (displaying aa 17-31 from HPV31) [36], MS2-31/16L2 VLPs (displaying tandem L2 peptide 20-31 and 17-31 from HPV31 and HPV16, respectively), MS2-consL2(69-86), MS2-consL2(108-122), MS2-consL2(108-122)/31L2/16L2, or immunized with a mixture of VLPs [Mixed MS2-L2 VLPs: 5µg MS2-consL2(108-122)/31L2/16L2 plus 5µg of MS2-consL2(69-86)]. All VLPs were mixed with alum hydroxide adjuvant prior to immunizations

and immunizations were done twice at two-week intervals. Anti-L2 IgG antibody responses in sera were tested by L2 peptide-ELISA using synthetic HPV16L2 (17-31), HPV31L2 (17-31), consL2(69-86), and consL2(108-122) as target antigens. The peptides were conjugated to streptavidin using biotin or SMPH [Succinimidyl 6-((beta-maleimidopropionamido)hexanoate)] and were then used to coat ELISA plates. ELISAs were conducted as described above and the titers of antibodies were determined as the reciprocal of the highest sera dilution with an OD₄₅₀ greater than 2-fold compared to that of control sera at the same dilution.

1.3.4 Pseudovirus production and purification

HPV PsV types (PsV: 16, 33, 45, 58) encapsidating a reporter plasmid [pCluf: encodes both green fluorescence protein (GFP) and luciferase] were produced in 293TT cells as previously described [39]. Briefly, 293TT cells were transfected with a mixture of two plasmids (each HPV shell and a pCluf). Forty-eight hours after transfection, cells were lysed and mature PsV was purified on a cesium chloride gradient by ultracentrifugation at 20,000 rpm for 17 hours or 40,000 rpm for 4 hours.

1.3.5 Cervicovaginal infection with HPV PsVs

Mice were intramuscularly immunized twice each with 5 µg of MS2 control VLPs, MS2 VLPs displaying L2 peptides or with Mixed MS2-L2 VLPs at two-week intervals. In addition to these, another group of mice was immunized with 5 µg of Gardasil-9. All immunizations were done with alum hydroxide adjuvant. Two weeks after the last immunization, mice were subcutaneously treated with 3mg of Depo-Provera. After five days, mice were vaginally challenged with $\sim 6.4 \times 10^6$ infectious unit (IU) of PsV. Forty-eight hours post-PsV challenge, mice were vaginally instilled with 0.4 mg of luciferin and imaged with a Caliper IVIS Lumina II as previously described [38].

1.3.6 *In vitro* neutralization assays

Neutralization assays were performed as previously described [40]. Briefly, HPV PsV18 and PsV33 encapsidating a reporter plasmid (p_{fwB}: expresses GFP) were made in 293TT cells as above. HPV PsV18 and PsV33 were titrated in pgsa-745 cells and virus titers that lead to 20% and 75% infectivity (expression of GFP relative to control cells), respectively, were used in the neutralization assay as followings: extracellular matrix (ECM) from MCF10A cells was deposited in 96-well plates followed by the addition of the PsVs. The ECM-PsVs were incubated overnight followed by the addition of serial-dilutions of sera collected from

immunized mice or addition of growth media as control. The plates were incubated for 6 hours and pgsa-745 cells were added. Forty-eight hours later, PsV infectivity/neutralizations were assessed by flow cytometry based on the expression levels of GFP in infected cells. The reciprocal of the highest sera dilution that inhibited 50% of PsV infection relative to control sera was considered the neutralization titer.

1.3.7 Statistical analysis

Statistical analyses for ELISA and PsV challenges were determined by unpaired two-tailed t-test and unpaired one-tailed t-test, respectively.

1.4 Results

1.4.1 Insertion of multiple L2 epitopes on MS2 coat protein does not affect VLP assembly

We had previously shown that immunization with MS2 bacteriophage VLPs displaying HPV16L2 (17-31) offered complete protection against HPV PsV16 but suboptimal cross-protection against other high-risk HPV PsV types. To enhance protection against these other HPV types, we explored several strategies. First, we constructed recombinant coat proteins displaying a tandem L2 peptide (aa

20-31 from HPV31 and aa 17-31 HPV16) or consensus L2 sequences (representing aa 69-86 or aa 108-122) at the N-terminus of the MS2 coat protein. The consensus sequence was obtained by aligning amino acid sequences from 19 HR-HPV types plus 4 low-risk types using Jalview software (version 2.9.0b2) and T-coffee program (Fig. 2). In a second strategy, we inserted multiple epitopes [consL2(108-122) and the tandem 31L2/16L2 peptide] on the MS2 coat protein.

Fig. 2. Consensus sequence of HPVs L2 proteins. Consensus sequences were obtained by aligning HR-HPV types (HPVs 16, 18, 26, 31, 33, 34, 35, 39, 45, 51-53, 56, 58, 59, 66, 68, 73 and 82) and low-risk HPV types (HPVs 6, 11, 43, and 44) using Jalview software (<http://www.jalview.org/>) and T-coffee program (an plugin of Jalview software). Highly conserved amino acids

(conservation) are shown in yellow/gold tall bars whereas less conserved amino acids are shown in tan/chocolate short bars. Glycine (G) at position 85 was not included in designing consensus (69-86) peptide because only HPV 11 had G at this position. “+” at position 114 was replaced with valine (V) since most HPVs have V at this site. Glutamic acid (E) at 116 and Alanine (A) at 121 were replaced with isoleucine (I) and proline (P), respectively.

The results of TEM analysis indicated that proteins produced from all these recombinant constructs assembled into VLPs (Fig. 3 and Table 1).

Fig. 3. Transmission electron microscopy (TEM) images of MS2-L2 VLPs that were assembled from recombinant proteins containing various L2 epitopes. All images were taken at magnification of 70,000X.

To confirm that the coat proteins contained L2, VLPs were analyzed by Western blot using anti-MS2 and anti-HPV16 L2 (aa 1-88) serum. As shown in Fig. 4A, anti-HPV 16L2 serum reacted with all VLPs except with MS2-consL2(108-122)

and control MS2 VLPs as expected. As expected, anti-MS2 serum reacted with each VLP. The varying sizes of recombinant MS2-L2 protein bands reflect the differences in the sizes of inserted L2 epitopes.

Fig. 4. Characterization of L2 peptides on MS2 VLP. A) Reactivity of HPV16 L2 (aa 1-88) serum and MS2 serum with MS2-L2 VLPs or MS2 VLPs in Western

blots. One hundred ng of VLPs were loaded to 10% SDS-PAGE gel and 16L2 (1-88) serum (top) and MS2 serum (bottom) were used as primary antibodies at a dilution of 1:5,000. B) Reactivity of HPV16 L2 (aa 1-88) serum with MS2-L2 VLPs or MS2 VLPs. ELISA plates were coated with 500 ng of VLPs and reacted with the serum at a dilution of 1:10,240 (left) and 1:640 (right). The average OD_{s450} for 4 mice are shown. Error bars stand for standard error of the mean (SEM).

To assess if the L2 peptides were actually displayed on intact VLPs, an ELISA was conducted using MS2-L2 VLPs or control MS2 VLPs as target antigens. As shown in Fig. 4B, anti-HPV16 L2 serum (at a 1:10,240 dilution) reacted with MS2-31/16L2 VLPs and MS2-consL2(108-122)/31L2/16L2 VLPs, and, to a lesser extent (at a 1:640 dilution) with MS2-consL2(69-86) VLPs. These data suggest that the peptides are displayed on the surface of VLPs.

1.4.2 MS2 VLPs displaying multiple L2 epitopes are immunogenic

To assess the immunogenicity of MS2 VLPs displaying the L2 peptides, mice were immunized with two doses (5 µg/dose) of purified MS2-L2 VLPs or control MS2 VLPs. Another group of mice was immunized twice with a mixture of VLPs [Mixed MS2-L2 VLPs; MS2-consL2(108-122)/31L2/16L2 plus MS2-consL2(69-86)] in order to assess whether immunizing with multiple L2 VLPs could enhance

the spectrum of protection. In general, all of the VLPs elicited high-titer antibody responses. Sera collected from mice immunized with MS2-16L2, MS2-31L2, MS2-31/16L2, and MS2-consL2(108-122)/31L2/16L2 VLPs had high antibody titers ($>10^4$) against HPV16 and HPV31 L2 peptides (Figs. 5A-B). Moreover, mice immunized with MS2-consL2(69-86) VLPs had high IgG titers against consL2(69-86) peptide (Fig. 5C). In contrast, VLPs displaying the consL2(108-122) peptide were less immunogenic and induced lower antibody titers ($<10^3$) (Fig. 5D). Mice immunized with Mixed MS2-L2 VLPs had similar antibody titers ($>10^4$) against HPV16 L2, HPV31 L2 and consL2(69-86) peptides as mice immunized with individual VLPs (Figs. 5A, B and C), suggesting that mixing the VLPs did not compromise the immunogenicity of individual L2 epitopes.

Fig. 5. Immunogenicity of MS2-L2 VLPs in mice. Mice were immunized twice with 5 μ g of MS2-L2 VLPs or control MS2 VLPs at two-week intervals. Sera were collected two weeks after the last immunization and anti-L2 peptide IgG titers were determined by end-point dilution ELISA using: A) 16L2 (17-31) peptide, B) 31L2 (17-31) peptide, C) consensus L2(108-122) peptide, and D) consensus L2(69-86) peptide as target peptides. Titers were determined as the reciprocal of highest sera dilutions at which reactivity of experimental sera was at least twice that of control MS2 sera. “Mixed MS2-L2 VLPs” serum was collected from mice immunized with a mixture of consL2(108-122)/31L2/16L2 and consL2(69-86) VLPs. Each datum (circles, squares, rectangles, and triangles, etc.) represents

titer in an individual mouse and lines stand for the geometric mean for each group.

1.4.3 MS2 VLPs displaying multiple L2 epitopes offered broad protection against HPV PsVs, at levels similar to Gardasil-9 vaccine

To determine whether immunization with MS2-L2 VLPs conferred protection from genital infection with various HPV types, immunized mice were vaginally infected with HPV PsV (16, 31, 45, and 58) and protection efficacies were compared to those of mice immunized with Gardasil-9 or control MS2 VLPs. We assessed cross-protection against HPV PsV31 and 58 because in a previous study [34] we observed suboptimal cross-protection against these PsVs following immunization with MS2-16L2 VLPs. Mice immunized with MS2-16L2, MS2-31L2, MS2-31/16L2, MS2-consL2(108-122)/31L2/16L2, and Mixed MS2-L2 VLPs showed protection against HPV PsV31, at levels similar to Gardasil-9 (which contains HPV31 VLPs) vaccine-immunized mice (Fig. 6A); Moreover, almost complete protection were observed against PsV58, PsV45, and PsV16 challenge (Figs. 6B-6D). Mice immunized with MS2-consL2(69-86) VLPs showed only slight protection (not significant) against the HPV PsV types tested, and MS2-consL2(108-122) was not protective. Mixed MS2-L2 VLPs offered the best protection against all PsV types except against PsV58.

A)

Mice infected with HPV PsV31

B)

Mice infected with HPV PsV58

C) Mice infected with HPV PsV45

D)

Mice infected with HPV PsV16

Fig. 6. Mice immunized with MS2-L2 VLPs elicit protective antibody responses against HPV PsV31, 45, 58, and 16. Mice vaccinated with MS2-L2 VLPs or MS2 control VLPs were vaginally challenged with $\sim 6.4 \times 10^6$ IU of HPV PsVs. Forty-eight hours post-challenge, luciferin was instilled vaginally and images were taken 3 minutes post-luciferin instillation except in 4A where images were taken 10 minutes post-luciferin instillation. The average radiance (p/s/cm²/sr) of luciferase expression at the genitals was determined using Living Image 3.2 software. Each datum represents the radiance value of an individual mouse and the lines stand for the average geometric mean for each group. Red dashed lines highlight MS2-L2 VLPs efficacy in comparison to Gardasil-9 vaccine.

The *in vitro* neutralization assay is an alternative method that can be used to test the neutralization efficacy (i.e. predict protection potentials) of candidate HPV vaccines. To test the neutralizing potential of our MS2-L2 VLPs with other HPV types, sera from mice immunized with VLPs (MS2-31/16L2 and Mixed MS2-L2) that offered the best protection against vaginal infection were used in an *in vitro* neutralization assay against PsV18 and PsV33. Compared to control MS2 sera, sera from mice immunized with MS2-31/16L2 or Mixed MS2-L2 VLPs neutralized PsV18 and PsV33 at levels similar to sera from mice immunized with Gardasil-9 (Fig. 7). These results show that MS2-31/16L2 and Mixed MS2-L2

VLPs sera are protective *in vivo* and *in vitro* against infection by diverse HPV types.

Fig. 7. MS2-L2 sera neutralize HPV PsV18 and PsV33. Titers of HPV PsV18 and PsV33 that led to 20% and 75% infectivity, respectively, of pgsa-745 cells were separately incubated with serial-dilutions of sera collected from mice immunized with MS2-L2 VLPs or controls. Incubated PsV and sera were then used to infect pgsa-745 cells. Forty-eight hours after infection, PsV neutralization (based on GFP expression levels) was determined by flow cytometry. Bars indicate the reciprocal of the highest sera dilution at which at least 50% HPV PsV was neutralized compared to controls with no sera. MS2 control sera neutralizing titers were <50.

1.5 Discussion

Current HPV vaccines protect mostly against the HPV types included in the vaccines [4]. Given the fact that protection from HPV infections is not 100%, patients vaccinated with any of the HPV vaccines are still recommended to continue cervical cancer screening. The recommended screening is very crucial for HIV/AIDS patients given the immunocompromised nature of their immune system, their propensity to be co-infected with up to 10 HR-HPV types [12, 13], and the risk for their HPV infections to progress to invasive cancer 6 to 15 years earlier than non-HIV patients [41, 42]. Thus, there is a need to develop an HPV vaccine with the potential to offer complete protection against HPV-associated cancers, especially in HIV/AIDS patients. We had previously reported that a thermostable bacteriophage MS2 VLPs displaying peptide 17-31 from HPV16 offered complete protection against HPV PsV16 and other HPV PsVs, but suboptimal protection against two important heterologous HPV types (HPV31 and HPV58) [34, 43]. To enhance protection against some of these heterologous HPV types, we generated for the first time, VLPs [MS2-31/16L2 and MS2-consL2(108-122)/31L2/16L2] displaying concatemers of two or three L2 epitopes, respectively. In addition to these, we developed VLPs [MS2-consL2(69-86), MS2-consL2(108-122)] displaying consensus sequences. Anti-HPV16 L2 serum reacted with all the MS2-L2 VLPs (confirming display of peptides on VLPs) except for VLPs displaying consL2(108-122) (Figs. 2A and 2B). Lack of reactivity with MS2-consL2(108-122) VLPs was expected because the HPV16 L2 (aa 1-88)

serum does not include consL2(108-122) epitope. The reactivity of anti-HPV16 L2 serum with MS2-consL2(69-86) VLPs (compared with other L2-VLPs) was low. This could be explained by the fact that HPV16 L2, from which the serum was generated is 80% identical to 31L2 (at aa 17-31) unlike peptide 69-86 of HPV16 L2, which is only 72% identical to consL2(69-86).

Mice immunized with VLPs displaying concatemers, consensus sequences [except MS2-consL2(108-122)], and Mixed MS2-L2 VLPs [MS2-consL2(108-122)/31L2/16L2 plus MS2-consL2(69-86)] elicited high anti-L2 antibody titers (Fig. 3), which protected/neutralized six HPV PsV types (16, 18, 31, 33, 45, 58) at levels similar to Gardasil-9 vaccine (Figs. 4 and 5). Mixed MS2-L2-immunized mice offered the best protection except against PsV58; we are not sure why partial protection was observed given the fact that 31L2 and 16L2 (17-31) share ~87% and 80% amino acid identity, respectively, with 58L2 at the same epitope. MS2-16L2 VLPs in this study offered good protection (although a little less compared to MS2-31L2 VLPs) against PsV31 and PsV58, whereas we had observed less protection in a previous study [34]. It is likely that mice in our previous study were challenge with higher PsV dose compared to this study. For example, control MS2 mice challenged with PsV31 in our previous study had a higher geometric mean average radiance (GMAR $10^{5.5}$) compared to MS2 control mice in this study with GMAR of $10^{4.8}$. Irrespective of these differences, these results suggest that immunization with a tandem L2 peptide or a mixture of L2 peptides does not compromise the immunogenicity of individual epitopes.

While robust responses were observed with the VLPs displaying peptide 17-31 (single or tandem peptide), little protection was observed in mice immunized with VLPs displaying peptide consL2(69-86) and no immune responses were observed in mice immunized with VLPs displaying peptide consL2(108-122). It was surprising that no significant protection was observed in mice immunized with the MS2-consL2(69-86) given the fact that mice had high anti-L2 antibody titers against this peptide. A previous study had shown that sera (at 1:1000 dilution) from mice immunized with Q β bacteriophage VLPs displaying a similar conjugated consensus L2 peptide neutralized HPV PsV31, 45, 58 [36]. The discrepancy in the results could be explained by the fact that the previous study used a slightly longer consensus sequence (65-85), which included 4 additional amino acids (GTGG) that are highly conserved amongst HPV types. We attempted here to insert (genetically) the same epitope on the coat protein of MS2; however, no expression of the fusion protein was detected (data not shown). With respect to consensus peptide 108-122, we think MS2-consL2(108-122) was not immunogenic because the peptide may not have been properly displayed on the VLPs; previous studies had shown that the isolated peptide contains neutralizing epitopes [25, 36]. Unfortunately, we could not confirm genetic display on MS2 due to lack of polyclonal antibodies that include peptide 108-122.

1.6 Conclusions

In summary, we have shown that inserting a concatemer of two or three L2 peptides on MS2 coat proteins does not affect the ability of the coat proteins to assemble into VLPs. Additionally, the L2 epitopes are displayed on the VLPs and immunizations with a mixture of MS2 VLPs displaying multiple L2 epitopes do not compromise the immunogenicity of each individual epitope on the VLPs. MS2 VLPs displaying the tandem L2 peptide, (20-31) from HPV31 and (17-31) from HPV16, cross-protected/neutralized selected HR-HPV PsV types, suggesting that this is an excellent approach to achieve broader protection against more HPV types. Moreover, mixing MS2-consL2(108-122)/31L2/16L2 with MS2-consL2(69-86) VLPs offered protection/neutralization at levels similar to Gardasil-9 vaccine, suggesting that these MS2-L2 VLPs are candidates for low valence yet broadly cross-protective HPV vaccines that should be assessed further for protection against more HPV types especially those that may be more common in HIV patients.

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Chapter 2

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

1.1 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 and at the oral region, the VLPs protect against HPV16, 35, 39, and 58. Including our colleague's data (data not shown), 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. 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.

1.2 Introduction

Human papillomaviruses (HPVs) are non-enveloped DNA viruses with more than 220 different types identified to date [1-3]. Approximately, 40 of these HPV types are transmitted sexually through anogenital-to-anogenital sex or anogenital-to-oral sex [4, 5], and 19 HPV types can cause cancers. Anogenital HPV infections are associated with cervical cancer, vaginal cancer, penile cancer, anal cancer, and genital warts [6-10] whereas oral HPV infections are associated with head and neck cancers (HNCs; oral, oropharyngeal, laryngeal cancers, etc.) including recurrent respiratory papillomatosis (RRP) [11-15]. Twenty-five percent of HNCs are caused by HPVs [16]; 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.) [11, 17-19]. 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 [17, 19]. Two prophylactic vaccines (Gardasil-9 and Cervarix; Gardasil-4 has been discontinued) are currently being used to protect against HPV infections [6, 20, 21]. 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. The 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 [22], 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 [23-26]. 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 [22]; 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, and 53) 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 [27-30]. We also assessed whether the mixed VLPs can be developed into a heat-stable dry powder formulation with mucosal adjuvants; we assessed the thermostability/immunogenicity of the dry powder VLPs stored at room temperature for 60 days.

1.3 Materials and Methods

1.3.1 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 [22]. The plasmids were used to transform C41 *Escherichia coli* (*E. coli*) cells. Protein expression and VLPs purification were conducted as previously described [22]. Briefly, transformed bacterial C41 culture was induced with 0.5 mM Isopropyl β -D-1-thiogalactopyranoside (IPTG) for 3 hours 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 CL-4B columns.

1.3.2 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 μ g) was further mixed with 2 μ g 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 MTDL excipient solution (containing Mannitol (M, 75% w/w), Trehalose (T, 7.5% w/w), Dextran (D, 2.5% w/w), and L-Leucine (L, 15% w/w). 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 [31, 32]. 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 (Q) between 10 and 15mm, 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) [31] 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.

1.3.3 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 [33]. 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.

1.3.4 Immunization of mice and assessing antibody responses

All animal work was conducted following Michigan Technological University Institutional Animal Care and Use Committee (IACUC) 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 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 SFD VLPs but with additional 2 µg each of fresh liquid CT and MPLA adjuvants; the latter will 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 [34]. 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 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 1M 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 μ L 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.

1.3.5 Oral and vaginal infection with HPV pseudoviruses (PsVs)

HPVs pseudoviruses representing PsVs 16, 35, 39, 53, and 58 were expressed and purified by cesium chloride gradient ultracentrifugation as previously described [26]. 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 (16 and 53) infection was conducted as previously described [22]. For oral infection, immunized mice were injected at the buccal region with PsVs (16, 35, 39 and 58); $1 - 7 \times 10^6$ 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/cm²/sr) was determined by drawing equally sized regions of interests surrounding the site of PsV instillation.

1.3.6 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.

1.4 Results

1.4.1 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 cholera toxin and MPLA in the floor of the mouth or by injecting the buccal area of the mouth with the VLPs. 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).

C) Genital protection from HPV PsVs

PsV 53

Fig. 1. Immunogenicity of mixed MS2-L2 VLPs with/without mucosal adjuvants (CT or MPLA) and protection from PsV16 and 53. Mice were immunized (buccal injection) thrice with 100 μ g 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 μ g of mixed MS2-L2 VLPs, control MS2 VLPs, and 5 μ g of Gardasil-9 vaccine (immunized intramuscularly). The mice were vaginally challenged with 1.6×10^6 IU of PsV 53. The average radiance (p/s/cm²/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.

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 [34, 35]. 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, which is not included in Gardasil-9 vaccine. Mice immunized with mixed MS2-L2 VLPs offered complete protection against PsV53 while Gardasil-9 offered no protection against PsV53 (Fig. 1C).

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

MS2-L2 VLPs offered best protection against PsV35 and PsV39 (Fig. 2B).

A)

B) Oral protection from HPV PsVs

Fig. 2. Buccal immunization with mixed MS2-L2 VLPs and oral protection from HPV PsVs 16, 35, 39 and 58. Mice immunized (buccal injection) thrice (at two-week intervals) with 100 μ g of mixed MS2-L2 VLPs, control MS2 VLPs, and 5 μ g of Gardasil-9 vaccine (immunized intramuscularly) were orally challenged with: A) 7×10^6 IU of PsV16 and B) $1 - 7 \times 10^6$ IU of PsVs 35, 39 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.

1.4.2 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).

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.

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).

A)

B)

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.

1.4.3 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 (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 Figs. 5B and 5C, 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).

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.

1.5 Discussion

HPV-associated HNCs are on the rise especially oropharyngeal squamous cell carcinomas (OPSCC), a type of HPV+HNC. The number of HPV-associated OPSCCs 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) [11, 13, 36, 37]. 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 [13]. 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. Gardasil-4, which protected against HPV6, HPV11, HPV16 and HPV18 has been shown to elicit oral IgG antibodies [38, 39], oral antibodies neutralize/protect against HPV6, HPV16, HPV18 [40, 41], and reduce oral prevalence of vaccine types (HPV types included in the vaccine) in vaccinees [42-44]. However, a protective effect of the

vaccine on HPV types not included in the vaccine was not observed. 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 [6, 45, 46]. 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 [6, 16, 18]. As an alternative to Gardasil-9, we developed a bacteriophage-based MS2-L2 VLP candidate vaccine against HPVs [22]. 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 [47]. 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) [48-50] and activates the mitogen activated protein kinase (MAPK) 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 [48, 49]. 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 (cholera toxin and MPLA), the protection level was enhanced; moreover, robust protection was observed when the two-adjuvant combinations were used in immunization (Figs. 1B & 1C); 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 [51-55]. 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, 58) associated with ~80% of HNCs (Figs. 2A and 2B). 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). 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 [56]; 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 assessed the potential of formulating mixed MS2-L2 VLPs together with cholera toxin and MPLA into a dry powder, with the ultimate goal of enhancing its thermostability of the candidate vaccine. We SFD the VLPs together with the 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 other studies, which showed that spray drying of vaccines with adjuvant (alum) lead to vaccine agglomeration [57]. 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 [22] 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 [22], we showed that the candidate vaccine offers protection from genital infection against six oncogenic HPV types (HPV16 18, 31, 33, 45, and 58). Our colleague has also shown that the candidate vaccine prevents oral infection from HPV 52 and genital infection from HPV 56 and HPV11 (data not shown). In the current study, we show that the candidate vaccine offers protection from genital with HPV PsVs 53 and protection from oral infection with HPV PsVs 16, 35, 39, 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 [22]. This view is supported by the fact that mice immunized with the mixed MS2-L2 VLPs, in our previous study [22], 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 [16-19]). 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 [6, 58]). The candidate vaccine also shows protection against HPV11 associated with 19-32% of recurrent respiratory papillomatosis [59, 60] and 36% of genital warts [61, 62]. 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 [6]; 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.

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Chapter 3

Novel Expression of Coat Proteins from Thermophilic Bacteriophage Φ IN93 and Evaluation for Assembly into Virus-like Particles

1.1 Abstract

The structural proteins of viruses can spontaneously assemble to form virus-like particles (VLPs) without the viral genome. VLPs are empty viral shells without the whole viral genome and not infectious. Overexpression of viral coat or envelope proteins in a suitable host cell allows the proteins to self-assemble into VLPs. VLP vaccines such as human papillomaviruses (HPVs) and hepatitis B virus (HBV), have been approved to protect against HPVs and HBV infections, respectively. In addition to this, a large group of researchers are working on adopting VLPs as a display platform to develop chimeric VLPs vaccines displaying heterologous antigens from other viruses, bacteria, parasites and even tumor-associated antigens. However, there are some challenges/limitations using current VLP display platform. For example, there are limitations on the size of a heterologous peptide that can be displayed on a VLP, the VLP not thermostable at room temperature, and pre-existing antibody to the VLP platforms may limit their use to elicit antibodies against heterologous antigens. To address some of these questions, especially thermostability and pre-existing antibodies, we

decided to explore if two coat proteins, open reading frame (ORF) 13 and ORF14, from a thermophilic bacteriophage, Φ IN93, can be expressed in *E. coli* and if the coat proteins can assemble into VLPs. Here, we co-expressed and purified ORF13 and ORF14 proteins from *E. coli*. Our results seem to suggest that co-expression of two truncated versions of the coat proteins have potential to form structures that resemble VLPs. To the best of our knowledge, this is the first attempt to develop a new thermophilic VLP platform based on a thermophilic bacteriophage.

1.2 Introduction

Viral genomes code for structural and non-structural proteins. Non-structural proteins include enzymes required for viral replication while structural proteins are protein components assembled around the viral genome to form a virus particle (virion) and include capsid (coat) proteins and in some cases envelope proteins [1]. The structural proteins by themselves can also assemble, naturally during the life cycle of a virion, without the viral genome to form structures, known as virus-like particles (VLPs) (reviewed in [2]). VLPs are therefore empty viral shells without the viral genome. VLPs can be generated by cloning and expression of the coat protein(s) or envelope proteins in a suitable host cell. Overexpression of coat proteins or envelope proteins in a host cell allows the proteins to spontaneously self-assemble into VLPs [3, 4].

VLPs can be derived from animal viruses, plant viruses, human viruses, and even from bacterial viruses (bacteriophages). VLPs have so many applications in science and in medicine; they can be used in pre-clinical studies to deliver drugs/cargo to specific cancer cells, to develop armored RNA (positive controls for diagnostic tests for infectious diseases), and for *in vivo* imaging (VLPs loaded with fluorophores). Furthermore, because VLPs resemble viruses in terms of size, multivalency of coat proteins on capsid, inability to replicate, and in terms of immunogenicity, they have can also be used to develop vaccines [5-9]. Two VLPs-based vaccines, derived from human papillomaviruses (HPVs) and hepatitis B virus (HBV), have been approved by the Food and Drug Administration (FDA) to protect against HPVs [10, 11] and HBV [2] infections, respectively; other VLPs-based vaccines against viruses are under development or in clinical trials [12, 13]. VLPs cannot only be used to develop vaccines against viruses from which the structural proteins are derived from but they can also be used as display platforms to develop chimeric VLPs vaccines displaying heterologous antigens from other viruses [14-18], bacteria [19], parasites [20, 21] and even tumor-associated antigens [22]. The goal of chimeric VLPs is to elicit immune response against the heterologous antigens on the platform. Thus, VLPs have been applied to develop candidate vaccines against both microbial and non-microbial diseases [23] ranging from allergy, hypotension, cholesterol [24], to cancer [22].

Despite the success of using VLPs as platforms to develop candidate vaccines, there are some challenges associated with current VLPs platforms. Some coat proteins have limitations on the size and the type of heterologous antigens that can be genetically inserted on the coat proteins without affecting the ability of the coat proteins to assemble into VLPs [5, 25, 26]. Additionally, some VLPs are not thermostable; in some cases, thermostability decreases with the insertion of heterologous antigens [27, 28]. Furthermore, most VLPs platforms used for peptide display (chimeric VLPs) including vector platforms, for vaccine design, are derived from viruses that infect humans and/or humans had previously been immunized with VLPs or antigens derived from these viruses. Thus, pre-existing antibodies against the VLPs display platforms in the general population, may compromise the immunogenicity of the display platforms and ultimately that of heterologous antigens displayed on the platforms. [29, 30]. To address some of these challenges, we assessed whether the coat proteins from a bacteriophage, Φ IN93, can assemble into VLPs. Bacteriophage Φ IN93 is a thermophilic virus isolated from a thermophilic bacterium, *Thermus aquaticus* with optimum growth temperature of 75°C [31].

Bacteriophage Φ IN93 is a double-stranded DNA virus with a circular genome size of ~19.4kbp. Two putative coat proteins, open reading frame (ORF) 13 and ORF14, have been identified in the bacteriophage based on sequence homology with the major coat proteins (VP16 and VP17) of another thermophilic bacteriophage, P23-77 [32]. ORF13 and ORF14 of thermophilic bacteriophage

Φ IN93 are 80% and 73% identical, respectively, to VP16 and VP17 of P23-77 (Fig. 1). It has been shown that a mixture of VP16, VP17, and VP11 (a minor capsid protein) of P23-77 can form complexes *in vitro* [33], which is a prelude to assembly/VLPs formation. However, no studies have ever assessed the expression of ORF13 and ORF14 from bacteriophage Φ IN93 or assessed the ability of these coat proteins to assemble into VLPs. Here, we assessed for the first time, the expression of ORF13 and ORF14 in a bacterial expression system and assessed the potentials of the coat proteins to assemble into VLPs with the long-term goal of developing a novel thermophilic bacteriophage VLPs platform for vaccine design.

Fig. 1. Sequence alignments of coat proteins of bacteriophages Φ IN93 and P23-77. Sequence alignment of: A) ORF13 of bacteriophage Φ IN93 & VP16 of bacteriophage P23-77 and B) ORF14 of bacteriophage Φ IN93 & VP17 of bacteriophage P23-77. Alignments were done using Clustal Omega. Asterisk (*) denotes identical amino acids, colon (:) denotes amino acids with similar properties and period (.) denotes amino acids with weak similar properties.

Amino acids highlighted in gray-background were used in model structures in Figure 4.

1.3 Materials and methods

1.3.1 Generation of expression vectors

The genes encoding potential coat proteins (ORF13 and ORF14) of bacteriophage Φ IN93 were codon-optimized for high-level protein expression in *E. coli* bacteria and were synthesized by Epoch Life Science. The complete sequence of each gene was separately amplified by polymerase chain reaction (PCR) and cloned into pET28a plasmid (Kanamycin resistance) and pET15b plasmid (Ampicillin resistance) using NcoI and BamHI restricted sites; this gave rise to four plasmids, namely: pET15b-ORF14, pET28a-ORF13, pET15b-ORF13, and pET28a-ORF14.

In addition to cloning the complete sequences of ORF13 and ORF14, we also generated truncated versions of each of the two genes. To generate truncated ORF13 and ORF14 genes, DNA sequences representing amino acids (aa) 21-165 and aa 46-271, respectively, were separately amplified by PCR and each truncated gene was cloned into the pET28a and pET15b using the same restriction sites above; this gave rise to four additional plasmids, namely: pET15b-ORF13-trunc with aa 21-165, and pET15b-ORF14-trunc with aa 46-271,

pET28a-ORF13-trunc with aa 21-165, and pET28a-ORF14-trunc with aa 46-271. In all cases, the genes in each of the constructs were sequenced across restriction sites to confirm the cloning.

1.3.2 Coat protein expression and purification

For coat protein expression, C41 *E. coli* cells were transformed separately with each plasmid and single colonies were screened for protein expression as follows. Bacterial cultures from colonies were grown in Luria-Bertani (LB) medium at 37 °C until the culture reached an optical density (OD)₆₀₀ of 0.6. Coat protein expression was induced with 0.5 mM Isopropyl β -D-1-thiogalactopyranoside (IPTG) overnight at the same temperature. For co-expression of ORF13 and ORF14 in the same bacteria, equal concentrations of pET15b-ORF14 and pET28a-ORF13 or pET28a-ORF14 and pET15b-ORF13 or pET28a-ORF13-trunc and pET15b-ORF14-trunc or pET15b-ORF13-trunc and pET28a-ORF14-trunc were mixed and used to transform C41 cells. Transformed cells were grown in LB medium with 30 μ g/ml of kanamycin plus 50 μ g/ml of ampicillin at the same temperature until the culture reached an OD₆₀₀ of 0.6. Coat protein expression was induced with 1 mM IPTG under the same conditions and time as above. To screen for coat protein expression, bacterial cells were pelleted, resuspended in 8M urea, sonicated and resolved on SDS-PAGE gel.

Cultures, which showed protein expression, were used to grow large volume cultures (100 ml or 250 ml) for large-scale protein expression and purification. The cultures were grown and induced as above. Cell pellets were then lysed using 0.2% lysozyme solution (100 mM NaCl, 10 mM EDTA, 50 mM Tris-HCl PH 8.5 plus 0.05% deoxycholate) and sonicated to help lyse the bacteria. Two ng/ul of DNase and 2 mM of MgCl₂ were added to the lysates and the lysates were incubated for 1 hour at 37 °C. Lysates were spun at 3,700 rpm (4 °C) for 30 minutes and the supernatants collected and resuspended in 50% ammonium sulfate to precipitate coat proteins. The supernatants with precipitated coat proteins were spun at 10,000 rpm (4 °C) for 10 minutes. The pellets were resuspended in sepharose column buffer (SCB: 10 mM Tris-HCl PH 7.4, 100 mM NaCl, 0.1 mM MgSO₄), spun at the same time and conditions. Supernatants were then loaded on sepharose cross-linked CL-4B column and fractions were collected. Fractionation tubes containing coat proteins were combined and the SCB buffer was exchange with PBS (1x phosphate buffered saline) using Millipore Amicon™ Ultra-4 Centrifugal Filter Units (100K MWCO).

1.3.3 Generation of bacteriophage Φ IN93

Φ IN93 was amplified and purified as previously described [31]. Briefly, 100 ul of overnight culture (70 °C) of *Thermus thermophilus* bacterial strain TZ2 was used to inoculate a fresh A2 medium (0.1% tryptone, 0.1% yeast extract, and

Castenholtz basal salts pH 7.5). The culture was grown at 70 °C for 4 hours and was subsequently inoculated with a plaque of bacteriophage Φ IN93. Then, the culture was shaking for another 2.5-3 hours until the media became clear (Lysis). The culture was spun down at 3000 rpm for 10 min and the supernatant which contained phage was saved.

1.3.4 Transmission electron microscopy (TEM)

To assess the potential of coat proteins to assemble into VLPs, TEM was conducted as follows. Purified coat proteins of bacteriophage Φ IN93 or the virus were loaded onto glow-discharged carbon grids for 2 minutes and then grids were stained with 2% uranyl acetate for 2 minutes. Samples were visualized at a magnification of 10,000 X.

1.3.5 Generation of sera for immunoassays

To generate sera that can react with both ORF13 and ORF14, we expressed ORF13 and ORF14 in pET28a as a recombinant protein with three amino acids, glycine-serine-serine, linker sequence in-between the two proteins. Expression and purification were done as described above. To generate sera, 3-4 week-old female Balb/c mice were immunized three times, intramuscularly, with 10 μ g of

purified recombinant protein (ORF13 + ORF14); all immunizations were done at two-week intervals in the presence of alum adjuvant. Two weeks after the last immunization, sera were collected and used for western blots against ORF13 and ORF14 coat proteins. Animal work was conducted in accordance with Michigan Tech Institutional Animal Care and Use Committee (IACUC) guidelines.

1.3.6 Western blots

One hundred ng of purified Φ IN93 coat proteins, bacteriophage Φ IN93, and control MS2 coat proteins were resolved on an SDS-PAGE gel and transferred to polyvinylidene fluoride (PVDF) membranes. The membranes were then blocked overnight with 5% non-fat milk followed by the addition of 1:1,000 dilution anti-ORF13 + ORF14 sera. The membranes were incubated for 2 hours at room temperature, washed, and 1:5,000 dilution of horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG antibodies were added for 1 hour at the same temperature. The membranes were then washed, developed with enhanced chemiluminescent substrate, and scanned using Luminescent Image Analyzer (LAS-4000).

1.4 Results

1.4.1 ORF13 and ORF14 can be successfully expressed in *E. coli*

To assess whether ORF13 and ORF14 coat proteins can be expressed in *E. coli*, given the fact that the coat proteins are derived from a thermophilic bacteriophage, we first generated expression vectors, pET15b-ORF14 and pET28a-ORF13, each with complete sequences of ORF14 and ORF13, respectively (Fig. 2). As shown in Fig. 2A, the coat proteins were successfully expressed in C41 *E. coli* cells. However, the expression level of ORF13 in pET28a seemed to be high compared to that of ORF14 in pET15b. To assess if the difference in expression level was due to the expression vectors used or the genes cloned to the expression vectors, we swapped the genes in the expression vectors by cloning ORF13 gene into pET15b and ORF14 gene into pET28a (Fig. 2B). The expression level of ORF14 from pET28a vector was still low compared to that of ORF13 from pET15b vector (Fig. 2A), suggesting that the gene and not the expression vector was associated with the differences in expression levels.

Fig. 2. Expression of coat proteins, ORF13 and ORF14, in pET15b and pET28a vectors in C41 cells. A) Plasmids pET15b-ORF14 or pET28a-ORF13 and B) plasmids pET15b-ORF13 or pET28a-ORF14 were used to transform C41 *E. coli* cells. The bacteria were grown at 37 °C and protein expression was induced with 0.5 mM IPTG. Cell pellets were lysed with 8 M Urea and loaded on SDS-PAGE gel for analysis. Gels were stained with Coomassie blue. Arrows indicate coat protein bands of ORF13 (18.81 kD) and ORF14 (32.01 kD). (-) is uninduced culture and (+) is induced IPTG culture. M=Molecular weight marker.

To assess whether ORF13 and ORF14 coat proteins can be expressed in the same bacteria, on the assumption that the two are required for assembly into

VLPs, we co-transformed C41 cells with two different combinations of expression vectors, pET15b-ORF14 and pET28a-ORF13 or pET28a-ORF14 and pET15b-ORF13. Both proteins were co-expressed in bacteria regardless of the combinations used (Fig. 3A). However, in pET15b-ORF14 and pET28a-ORF13 combination, ORF13 was expressed at high levels compared to ORF14 (results are similar to expression of the proteins, separately, above). In pET28a-ORF14 and pET15b-ORF13 combination, both proteins seemed to be expressed at similar levels (with ORF14 expression slightly higher). To assess whether ORF13 and ORF14 coat proteins (complete sequences) can assemble into VLPs, TEM analysis was conducted using co-expressed coat proteins. Unfortunately, none of the expressed coat proteins from the plasmid combinations, pET15b-ORF14 and pET28a-ORF13 or pET28a-ORF14 and pET15b-ORF13, assembled into VLPs. To confirm that the purified coat proteins were proteins of interest, we did a western blot using anti-ORF13 + ORF14 antibodies. As shown in Fig. 3B, the antibodies reacted with both proteins as well as with our positive controls, ORF13 and ORF14, from the bacteriophage Φ IN93.

Fig. 3. Co-expression of ORF13 and ORF14 proteins in C41 cells. A) Equal concentrations of pET15b-ORF14 and pET28a-ORF13 (Co-1) or pET15b-ORF13 and pET28a-ORF14 (Co-2) were mixed together and the mixtures were then used to separately transform C41 *E. coli*. The bacteria were grown at 37 °C and co-protein expression was induced with 1 mM IPTG. Cell pellets were lysed with 8 M Urea and loaded on SDS-PAGE gel for analysis. Gels were stained with Coomassie blue. Arrows indicate coat protein bands of ORF13 (18.81 KD) and ORF14 (32.01 KD). (-) is uninduced culture and (+) is induced IPTG culture. M=Molecular weight marker. B) Western Blots: ORF13, ORF14 and co-expressed proteins (ORF13 and ORF14) were prepared for SDS-PAGE gel and detected by serum raised from mice immunized with ORF13 + ORF14 proteins. Bacteriophage MS2 VLPs were used as a negative control while the purified ORF13-ORF14 recombinant proteins were set as positive control.

1.4.2 Truncated ORF13 and ORF14 can be co-expressed and purified from *E coli*

As mentioned above, ORF13 and ORF14 is 80% and 73% identical, in terms of amino acid, to VP16 and VP17 of bacteriophage P23-77, respectively. The 3D structure of VP16-VP17 complex has been predicted; VP16 forms a homodimer in the complex and VP17 coat protein interacts with at least one monomer of VP16 in the complex [34]. Given this information, we decided to

predict the 3D structure of ORF13-ORF14 complex, based on structure of VP16-VP17 complex, using SWISS-MODEL and Cn3D [35, 36]. As shown in Fig. 4, the 3D structure of ORF13-ORF14 complex is similar to that of VP16-VP17; the software predicted the structure using only amino acids number 21-165 from ORF13 and amino acids 46-271 from ORF14 (the same range of amino acids for VP16-VP17 complex). Because the structure was predicted using only amino acids 21-165 and 46-271 but not the complete sequences, we generated truncated versions of ORF13 (aa 21-165) and ORF14 (aa 46-271) and assessed the potential of the coat proteins to assemble into VLPs. ORF13-trunc and ORF14-trunc were successfully expressed, as single proteins or co-expressed proteins, regardless of whether pET15b or pET28a was used as the expression vectors (Figs. 5A and B). Purified single-expressed ORF13-trunc coat protein but not the single-expressed ORF14-trunc reacted with the recombinant ORF13-ORF14 antibodies (Fig. 5C); these results including those of co-expressed ORF13-trunc and ORF14-trunc are consistent with the results of coat proteins expressed with complete sequences above (Fig. 3B).

Fig. 4. Three dimensional (3D) structures of coat proteins of bacteriophages Φ IN93 and P23-77. The 3D structure of ORF13-ORF14 complex (left) from bacteriophage Φ IN93 was modeled, based on sequences and the 3D structure of VP16-VP17 complex (right; Cn3D view) from bacteriophage P23-77 as template, using Swiss-Model software. Homodimers of ORF13 (middle) and VP16 (middle) interact with each of the two copies of ORF14 and VP17, respectively. Both structures were modeled using amino acids 21-165 from ORF13 or VP16 and amino acids 46-271 from ORF14 or VP17 (highlighted in gray-background in Figure 1).

A.

B.

C.

Fig. 5. Single-expression and co-expression of truncated ORF13 (ORF13-trunc) and truncated ORF14 (ORF14-trunc) proteins in C41 cells. A) Plasmids pET15b-ORF14-trunc or pET28a-ORF13-trunc were used to separately transform C41 *E. coli* cells and the bacteria were grown at 37 °C. Protein expression was induced with 0.5 mM IPTG. B) Equal concentrations of plasmids pET15b-ORF14-trunc and pET28a-ORF13-trunc (Co-3) or pET15b-ORF13-trunc and pET28a-ORF14-trunc (Co-4) were mixed and used to transform C41 *E. coli* cells. The bacteria were grown at 37 °C and co-protein expression was induced with 1 mM IPTG. Cell pellets were lysed with 8 M Urea and loaded on SDS-PAGE gel for analysis. Gels were stained with Coomassie blue. Arrows indicate protein bands, ORF13-trunc (16.06 kD) and ORF14-trunc (24.97 kD). (-) is uninduced culture and (+) is induced IPTG culture. M=Molecular weight marker. C) Western blots: ORF13-trunc, ORF14-trunc and co-expressed proteins (ORF13-trunc and ORF14-trunc) were prepared for SDS-PAGE gel and detected by serum raised from mice mentioned above. The phage MS2 VLPs were used as a

negative control while the purified ORF13-ORF14 recombinant proteins and the phage Φ IN93 were used as the positive controls.

1.4.3 Truncated ORF13 and ORF14 form structures that resemble VLPs

To assess whether the expressed coat proteins can assemble into virus-like particles, TEM analyses were conducted using purified coat proteins. As shown in Fig. 6A, single-expressed ORF13-trunc or ORF14-trunc gave rise to very small oval structures. The co-expressed version of the truncated coat proteins seemed to form structures that resemble VLPs (Fig. 6 B); their sizes (~75 nm to ~100 nm) were closer to those of bacteriophage Φ IN93 (Fig. 6C).

Fig. 6. Assessing the assembly of Coat proteins of single-expressed and co-expressed ORF13-trunc and ORF14-trunc. A) TEM of purified ORF13-trunc and purified ORF14-trunc. B) TEM of purified co-expressed truncated coat proteins (Co-3: ORF13-trunc and ORF14 trunc expressed from pET28a and pET15b, respectively) or (Co-4; ORF13-trunc and ORF14 trunc expressed from

pET15b and pET28a, respectively). C) TEM of bacteriophage Φ IN93. TEM were conducted at 25,000 X magnification.

A.

B.

Fig. 7. PCR amplifications of co-expressed ORF13 & ORF14 (complete gene sequences and truncated versions). A) Induced cultures from Figure 3A (Co-1 and Co-2 with complete gene sequences) were use as templates for PCR. The presence of ORF13 or ORF14 was tested using primers for each gene. Sizes of

ORF13 and ORF14 are ~579 base pairs and ~876 base pairs, respectively. B) Induced cultures from Figure 5B (Co-3 and Co-4 with truncated versions of genes) were used as templates for PCR. The presence of ORF13 or ORF14 was tested using primers for each gene. Neg: untransformed *E. coli* culture negative control. Sizes of ORF13-trunc and ORF14-trunc are ~441 base pairs and ~684 base pairs, respectively.

1.5 Discussion

Two coat proteins, ORF13 and ORF14, from a thermophilic bacteriophage Φ IN93 have 80% and 73% amino acid identity, respectively, with two major coat proteins, VP16 and VP17, of thermophilic bacteriophage P23-77 [32]. While VP16 and VP17 coat proteins have been expressed in previous studies [33, 34]. The expressions of ORF13 and ORF14 have never been explored. Here we cloned and assessed the expression of ORF13 and ORF14 individually and in combination (co-expression) in *E. coli* C41 cells with the long term-goal of assessing the potential of the coat proteins to assemble into virus-like particles (VLPs). ORF13 and ORF14 coat proteins were successfully expressed, as separate proteins, in C41 cells. Moreover, the coat proteins could also be co-expressed in the same bacteria by using two plasmids that are incompatible (pET28a and pET15b; have the same origin of replication, pBR322) by simply applying two different selective pressures (two antibiotics resistance; kanamycin

and ampicillin) on the bacterial cells for 24 hours. The co-expression of ORF13 and ORF14 using two incompatible plasmids are consistent with previous studies that have shown that two proteins can be co-expressed from incompatible plasmids by using different antibiotic to the vectors [37, 38]. While ORF13 and ORF13 were successfully expressed (individually or together), the expression of ORF14 in all cases was less compared to that of ORF13, regardless of whether the protein was expressed using pET28a plasmid or pET15b plasmid. In the co-expression study, especially Co-1 with pET28a expressing ORF13 and pET15b expressing ORF14, we initially thought the expression bacteria might only have pET28a-ORF13 plasmid (i.e. pET15b-ORF14 plasmid might have been lost from the bacteria due to plasmid incompatibility). To assess if both expression plasmids were maintained in the bacteria during expression, bacterial cultures expressing the coat proteins were induced and aliquots (equal volumes) of the cultures were used as templates for polymerase chain reaction (PCR). As shown in Fig. 7A, the band intensities suggest that both plasmid combinations (pET15b-ORF14 and pET28a-ORF13 or pET15b-ORF13 and pET28a-ORF14) were maintained in the bacteria. These data suggest that ORF14 expression may be normally low on the virus. This view is supported by our Western blot data using Φ IN93 virus. In our Western blot data (Figs. 3B and 5C), ORF14 band in the lane with positive control Φ IN93 virus is very faint compared to ORF13 band. The view (low expression) is further supported by data from a related coat protein, VP17 from P23-77, which is 73% identical to ORF14; in a Master's Thesis,

University of Jyväskylä (Finland), it was observed that VP17 was expressed at low levels compared to VP16 (80% identical to ORF13) following co-expression of VP17 and VP16 using a bicistronic VP16-VP17 transcription unit [39]. Taken together, our data suggest that ORF14 is expressed at low levels compared to ORF13. It is likely that less proportion of ORF14 may be required for viral assembly compared to ORF13 as has been suggested for related proteins, VP17 (540 copies) and VP16 (1080 copies) in bacteriophage P23-77 [33, 34]. We assessed whether ORF13 and ORF14 can assemble to VLPs. As mentioned in the introduction, a mixture of VP16, VP17, and VP11 (a minor capsid protein) of P23-77 has been shown to form complexes *in vitro* suggesting that these coat proteins may form VLPs. However, a sequence in bacteriophage Φ IN93 that is homologous to VP11 (minor capsid protein of P23-77) has not been identified, suggesting that the capsid protein of Φ IN93 may be composed of only two coat proteins, ORF13 and ORF14. This may be not surprising given the fact that in some viruses (e.g. *adeno-associated virus* type 2), only two coat proteins out of 3 or more coat proteins are sufficient to form VLPs [4, 40]. Unfortunately, none of the expressed ORF13 and ORF14 coat proteins (individually or together) assembled into VLPs.

Given the fact the expressed ORF13 and ORF14 coat proteins did not assemble into VLPs, we generated truncated forms of the proteins based on regions of the proteins (amino acid sequence ranges) that were used by Swiss-Model software to predict ORF13-ORF14 structure in Fig. 4 (left). To our

surprise, the truncated versions of the proteins (individually or co-expressed) seemed to be expressed at low levels compared to the full-length proteins. PCR result based on band intensities (Fig. 7B) showed that the expression plasmids for ORF13 and ORF14 were both maintained in the bacteria. We assessed whether the truncated versions of ORF13 and ORF14 can assemble to VLPs. We observed structures that look like VLPs but future studies need to confirm if these are actually Φ IN93 VLPs.

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Summary

Most of our work focused on developing HPV L2 candidate vaccines based on bacteriophage MS2 VLP platform and we also spent time assessing the thermostability of the candidate vaccine by using spray-freeze drying technique; furthermore, we assessed the potential of developing a novel VLP display platform based on a thermophilic bacteriophage Φ IN93. In chapter 1, we showed that inserting a concatemer of two L2 peptides (HPV31 L2 20-31 and HPV16 L2 17-31) on bacteriophage MS2 VLPs does not affect the ability of MS2 coat proteins to form VLPs. Mixed MS2-L2 VLPs (MS2 VLPs displaying the concatemer L2 epitope and MS2 VLPs displaying a consensus L2 epitope) elicited antibodies that cross-protected/neutralized selected HR-HPV pseudovirus types (HPV16, 31, 45, 58, 33 and 18), suggesting that this is an excellent approach to achieve broader protection against more HPV types. In chapter 2, we continued with the assessment of cross-protection of candidate vaccine against additional HPV types (HPV35, 39, 53, and 58). We showed that the candidate vaccine offers protection from genital infection with more HPV PsVs (HPV53) and protection from oral infection with selected high-risk HPV PsVs (HPV35, 39 and 58). Overall, in Chapters 1 and 2, the candidate vaccine showed superior protection, especially with HPVs (HPV35 and HPV39) compared to Gardasil 9. In Chapter 2, the candidate vaccine without adjuvants was successfully spray-freeze dried into powder for thermostability test. SFD did not affect VLP assembly and the immunogenicity. Moreover, after 60 days

storage in room temperature, the candidate vaccine still elicited high immunogenicity and protection against oral infection of HPV16 PsV. In the future, more high-risk HPVs types should be tested to assess the cross-protection potential of this candidate vaccine. Also, longer storage time such as six months to 1 year (at room temperature or higher) should be done to assess the thermostability of the VLPs. The longevity of immune responses of the candidate vaccine should also be tested.

In chapter 3, we successfully co-expressed and purified full-length and truncated coat proteins of Φ IN93 phage. VLP-like structures were observed in the co-expression of truncated coat proteins. However, this result needs to be further confirmed. One possible way is the gold nanoparticle staining. In brief, the VLP-like structures should be immuno-stained with serum (recombinant ORF13-ORF14) mentioned above in chapter 3 and then incubated with the secondary antibodies conjugated with gold nanoparticles. If they are VLPs formed by Φ IN93 coat proteins, the VLPs-primary antibody complex will be bound by the gold particles. This can be observed using a TEM. A positive control can be phage Φ IN93 which is supposed to be bound properly, while a negative control can be HPV pseudovirus 16 VLPs which is not expected to be bound by the antibody. However, if they fail to form VLPs in *E. coli*, another expression system, thermophilic bacterium (*Thermus aquaticus*), can be used to co-express and assess VLP assembly. The thermophilic bacterium might have some “elements”

to help the coat protein fold and re-assemble given the fact that this is the natural host of phage Φ IN93.

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Chapter 1 and Chapter 2 involve texts and figures originally published in the journal, Antiviral Research, by Lukai Zhai *et al.* in 2016, 2017 and 2019.

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Title: A novel candidate HPV vaccine: MS2 phage VLP displaying a tandem HPV L2 peptide offers similar protection in mice to Gardasil-9

Author: Lukai Zhai, Julianne Peabody, Yuk-Ying Susana Pang, John Schiller, Bryce Chackerian, Ebenezer Tumban

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Title: 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

Author: Lukai Zhai, Rashi Yadav, Nitesh K. Kunda, Dana Anderson, Elizabeth Bruckner, Elliott K. Miller, Rupsa Basu, Pavan Muttal, Ebenezer Tumban

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