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RESEARCH ARTICLE

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The h4 coil surface region of human papillomavirus type 58 L1 virus-like particle serves as a potential location for presenting the RG1 epitope peptide

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ABSTRACT

The licensed prophylactic human papillomavirus (HPV) vaccines, based on L1 virus-like particles (VLPs), effectively prevent infection and HPV-associated cancers caused by the vaccine types but offer limited protection against non-vaccine types. L2 N-terminal peptides, such as the RG1 epitope peptide, contain conserved cross-neutralizing epitopes, and their immunogenicity could be enhanced via display on the surface of L1VLPs. To our knowledge, there have been no reports on the construction and immunogenicity research of chimeric L1-L2 proteins based on HPV58 L1VLP, the third most prevalent high-risk type in Asia. Here, we inserted the RG1 epitope peptides at two sites of the highly expressed HPV58 L1 – the h4 coil region or the DE loop (with linkers) – to construct seven chimeras. These chimeras were expressed in insect cells, self-assembled into chimeric VLPs (cVLPs), and their immunogenicity was assessed in a mouse model. Notably, three cVLPs with h4 coil insertions elicited comparable levels of L1-specific antibody response in mice to the L1VLP control and induced cross-neutralizing antibody responses against fourteen pseudoviruses. Conversely, four cVLPs with DE loop insertions induced significantly lower L1-specific antibody titers compared with the L1VLP control ($p < .001$). This might be attributed to the disruption or obstruction of neutralizing epitope(s) targeted by HPV58-specific conformation-dependent monoclonal antibodies, caused by the sequence insertions. Our findings suggest that the h4 coil region of HPV58 L1VLP might be a potential location for RG1 epitope display, guiding the presentation of heterologous epitopes to develop chimeric HPV58 L1VLP-based vaccines.

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Introduction

Human papillomaviruses (HPVs), comprising almost 450 distinct types (genera *α, β, γ, μ, ν*), are responsible for 5% of all human cancers, as well as substantial precancerous and benign lesions.[1,2] Among these, cervical cancer is the fourth most common cancer among women worldwide, with an estimated 602,147 new cases and 341,831 deaths per year.[3] Infection with high-risk HPVs (hrHPVs), including over 20 identified types such as HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 68, is the primary cause of nearly all cervical cancers and a proportion of other anogenital and oropharyngeal cancers.[3] Low-risk HPV types such as HPV6, 11 (genus α10) are the main causative agents for condyloma acuminatum and recurrent respiratory papillomatosis.[4] Cutaneous HPVs like HPV27, 57 (genus α4) are common in cutaneous warts, and HPV5 (genus β1) is associated with epidermodysplasia verruciformis-related squamous cancers.[3,5] Hence, the burden of diseases linked to HPV infection is substantial, particularly in underdeveloped regions where over 85% of these diseases occur.[3]

The capsid of HPV is comprised of major capsid protein L1 and minor capsid protein L2, arranged in a $T = 7$ icosahedral formation.[6] The surfaces of HPV capsids are composed of relatively hypervariable loop structures, which serve as the primary target for type-specific neutralizing antibodies.[7,8] L1 can self-assemble into virus-like particles (VLPs), which retain the structural characteristics of the native virions, exhibiting an orderly, repetitive, and densely arrayed surface epitopes arrangement, and are capable of eliciting potent and type-specific neutralizing antibody (nAb) responses in vivo,[9] effectively preventing virion attachment to either the basement membrane or cervical epithelial cell surface.[10,11] Thus, L1VLPs are the primary component of licensed HPV vaccines, including Cervarix (HPV16, 18), Walrinvax (HPV16, 18), Gardasil (HPV16, 18, 6, 11), Gardasil-9 (HPV16, 18, 6, 11, 31, 33, 45, 52, 58), and Cecolin (HPV16, 18). Although these vaccines were proven to effectively prevent almost 100% of infections caused by vaccine types,[12–15] they provide limited protection against non-vaccine types. The current strategy for broadening vaccine coverage primarily emphasizes multivalent L1VLP combined immunization, as exemplified by Gardasil-9, which includes L1VLPs from seven high-risk and two low-risk types.[12] Similarly, a 14-valent vaccine developed by Sinocelltech incorporating an additional five high-risk types has successfully completed its Phase III clinical research. Nevertheless, the ongoing increase in the types of L1VLPs within a vaccine inevitably escalates both the complexity and cost of manufacturing, thereby posing significant challenges to the development and realization of polyvalent L1VLP vaccines aimed at providing comprehensive protection against all high-risk HPV types and other medically significant types.

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The N-terminus of L2 has been reported to contain conservative type-common epitopes capable of inducing a low level of cross-neutralizing antibodies in animals,[16–20] making it the focus of investigation for second-generation broad-spectrum vaccines. However, the immunogenicity of L2 linear-epitope peptides is relatively weak with respect to L1. To enhance the immunogenicity of L2 peptides, different strategies have been performed,[21–32] and a promising method is the multimeric display of L2 epitopes on surface regions of L1VLPs.[21–29] HPV16 and HPV18 are the most prevalent types globally, and the epitope regions of the corresponding L1VLPs have been effectively characterized through the recognition of monoclonal antibodies.[7,33] Subsequently, several chimeric HPV L1-L2 VLPs (cVLPs) based on either HPV16 or HPV18 L1VLPs have been documented in scientific literature.[21–29] Consistently, published findings have demonstrated the dual capability of L1-L2 cVLPs, effectively maintaining the significant advantage of inducing long-lasting L1-specific protection while also possessing the capacity to generate L2-specific cross-protection.[23,34,35] For instance, the protective capacity of the HPV18 L1-L2 cVLPs remained consistent when combined with HPV16 and HPV18 L1VLPs in a vaccine formulation, and meanwhile the efficacy of HPV16 and HPV18 L1VLPs against PsV16 appeared potentially enhanced by the addition of the HPV18 L1-L2 cVLPs in the vaccine.[23]

For HPV16 and HPV18 L1VLPs, the DE loop and the h4 coil region frequently served as the sites for inserting L2 epitopes, such as incorporating L2 peptides of HPV16, 31, 58 into HPV16 L1 DE loop or h4 coil, or L2 peptides of HPV33, 58 into HPV18 L1 DE loop or h4 coil, to create chimeric L1-L2 VLPs.[21–24] These cVLPs successfully induced non-inferior L1-specific neutralization antibody responses compared to those of L1VLPs in animals and also induced enhanced L2-specific cross-protection responses. However, the insertion of epitope peptides into these regions can also disrupt epitopes important for HPV16 and 18. For instance, insertion of HPV45 L2 epitope into the DE loop of HPV18 L1 led to a loss of binding capability with H18.J4 neutralizing monoclonal antibodies (mAbs),[25] and the incorporation of HPV16 L2aa.108–120 into the DE loop of HPV16 L1 resulted in the loss of the epitopes recognized by the V5 and E70 neutralizing mAbs.[26] Furthermore, the inserted regions as well as the inserted amino acid sequences appear to be a limitation for VLP assembly, which is critical for its immunogenicity.[26,35,36] For instance, the insertion in h4 helix either with 16L2aa.17–36 or 16L2aa.108–120 could both disrupt the assembly of HPV16 L1.[26,36] Thus, the approaches of incorporating L2 epitope peptides into another type of HPV L1 to construct new chimeric L1-L2 proteins may encounter some challenges, such as inefficient antigen display and the limited structural capacity of L1 surface loops to accommodate foreign epitopes, and potentially significant disruption of L1 oligomeric structures. To our knowledge, there have been no reports to date on the construction and immunogenicity research of L1VLP-based chimeric proteins of HPV58, which is one of the most prevalent high-risk types in cervical cancer in southern China, ranking second only to HPV16 and/or HPV18.[37]

The region spanning amino acids 17 to 36 of HPV16 L2 is conserved among many HPV types and stands out as the dominant neutralizing epitope region among the identified L2 neutralizing epitopes.[19] This specific segment is recognized by the mAb RG1, which exhibits excellent cross-neutralization against various HPV types, and thus is called RG1 epitope peptide whose core sequence is aa.21–30.[38] Here, we constructed 58L1-16L2 chimeras to explore potential position of HPV58 L1VLP for RG1 epitope display. Among the seven constructs, three with h4 coil insertions, H4/L2-A, H4/L2-B, and H4/L2-C, were demonstrated to have an excellent ability in inducing comparable levels of L1-specific IgG and neutralizing antibody in mice to those of L1VLP control, as well as a broad-spectrum of L2-specific neutralizing antibody capable of cross-neutralizing 14 types of tested HPV PsVs in vitro. The other four constructs with DE loop insertions induced significantly reduced levels of L1-specific IgG and neutralizing antibodies (nAbs) compared with the L1VLP control group. This observed reduction is likely attributed to the disruption or alteration of epitope(s) due to the sequence insertion. Therefore, our findings suggest that the h4 coil region of HPV58 L1 could potentially serve as a favorable option for inserting the RG1 epitope, providing guidance for the presentation of other L2 epitopes or heterologous epitopes across other pathogenic microorganisms to develop chimeric vaccines based on HPV58 L1VLP.

Materials and methods

Construction of recombinant baculovirus

The HPV58 L1 gene previously reported by our laboratory was utilized as the backbone.[39] The gene was Sf9 codon optimized, with truncation of N-terminal 4 residues and C-terminal 25 residues, and demonstrated high expression levels in the baculovirus system. The HPV16 L2 RG1 epitope peptide, in the form of either long or short peptides, was genetically engineered into the h4 coil region or the DE surface loop (with peripheral amino acid modifications) of HPV58 L1 using an overlapping polymerase chain reaction (PCR) strategy (Table 1). The resulting amplified PCR products were then inserted into pFastBac1 vector (BamH I/EcoR I restriction sites) to construct recombinant plasmids, which were subsequently used to transform DH10Bac E. *coli* cells (Invitrogen, Cat.: 10361–012) for preparation of recombinant bacmids. These recombinant bacmids were then transfected into Sf9 cells (Gibco™, Cat.: 11496015) following the Bac-to-Bac protocol (Invitrogen) for amplification of the recombinant baculovirus.

Expression and purification of chimeric 58L1-16L2 proteins

Sf9 cells were infected with recombinant baculovirus carrying chimeric 58L1-16L2 genes for a duration of 3 days, after which the cell lysates were extracted and purified by cesium chloride (CsCl) ultracentrifugation.[21,22,39] Briefly, lysates were subjected to ultracentrifugation on CsCl density gradients (1.23 g/mL) for 20 h, and the turbid band in the middle of

Table 1. Summary of the construction of chimeric HPV58 L1-16L2 genes and regions where L2 epitopes were displayed.

Chimeric gene	Backbone*	Region	Amino acid position of L2	Inserted L2 epitopes and amino acid linker
DE/L2-A	HPV58 L1	loop D-E	aa.133–137	AGP- HPV 16L2aa.17–38
DE/L2-B	HPV58 L1	loop D-E	aa.133–137	AGP- HPV 16 L2aa.18–32
DE/L2-C	HPV58 L1	loop D-E	aa.135–140	GPA- HPV 16 L2aa.17–38
DE/L2-D	HPV58 L1	loop D-E	aa.135–139	GPA- HPV 16 L2aa.18–32
H4/L2-A	HPV58 L1	h4 coil	aa.429–432	HPV16 L2aa.18–38
H4/L2-B	HPV58 L1	h4 coil	aa.429–432	HPV 16 L2aa.18–32
H4/L2-C	HPV58 L1	h4 coil	aa.429–432	HPV 16 L2aa.19–31

*The HPV58 L1 backbone was *N*- and C-terminal truncated L1 generated by our lab ([35]).

the tube was collected and dialyzed against 0.5 M NaCl-PBS at 4°C for 3 h, followed by a 5% sucrose (w/w)/60% CsCl (w/w) gradient ultracentrifuged. Subsequently, the CsCl fraction was collected for an additional 20 hours of CsCl density gradient ultracentrifugation. The turbid band was then collected and dialyzed against PBS at 4°C for 3 days. Following quantification using the Bradford assay, each protein sample (5 μg) was loaded onto a 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and stained with Coomassie blue.

Western blotting

The lysates or purified proteins were calculated by Bradford assay and then analyzed by SDS-PAGE with Coomassie blue staining or Western blot. Expression of chimeric L1-L2 proteins was verified by mAb Camvir-1 raised against HPV16 L1 (1:8000, Millipore, Cat.:CBL402-K). To verify the antigenicity of L2 epitopes, samples were probed with polyclonal rabbit sera raised against HPV16 L2 aa.17–40-KLH peptides. HPV16 L2aa.11–200 coupled with the FliC protein of E. *coli* was used as a control. HRP-labeled goat anti-mouse (1:3000, CWBIO, Cat.: CW0102S) or goat anti-rabbit IgG (1:3000, CWBIO, Cat.: CW0103S) was used as the secondary antibody.

Dynamic light scattering (DLS) and transmission electron microscope (TEM)

The purified proteins (100 μg/ml in PBS) were subjected to DLS and TEM analysis.[21,22,40] DLS measurements were performed on a Malvern Zetasizer Nano ZS (Malvern). Samples were equilibrated to 25°C prior DLS analyzed and the Z-average hydrodynamic diameter and polymer dispersity index (PDI) were determined from three independent measurements. For TEM analysis, purified proteins were adsorbed on a carbon-coated grid for 1 min, rinsed with distilled water, negatively stained with 1% uranyl acetate for 3 min and then examined with a TEM-1400 electron microscope (JEOL) operating at 80 kV with a magnification of 80,000 ×.

Enzyme linked immunosorbent assay (ELISA)

Cross-neutralizing L2 epitopes displayed on cVLPs were detected using ELISA, following the previously described protocol with minor modifications.[21,22,24] Briefly, 58L1-16L2 cVLPs (100 ng/well) were added to a 96-well plate (Costar, Cat.:RF1173) and adsorbed at 4°C overnight. Controls included HPV58 L1VLP, HPV16 L2 aa.17–40-KLH, and PBS. After blocking unbound sites with 5% (w/v) bovine serum albumin (BSA) in PBST (PBS +0.05% Tween20), anti-16L2 aa.17–40-KLH rabbit serum (100 μl/well, diluted at 1:3000) was added and incubated at room temperature for 2 h, followed by incubation with HRP-conjugated goat anti-rabbit IgG (diluted at 1:3000, CWBIO, Cat.: CW0103S) at 37°Cfor an hour. Enzyme activity was revealed with substrate O-phenylenediamine (Sigma-Aldrich, Cat.: P9029), and the reaction was stopped by adding 50 μl of 2 M H_2SO_4. The absorbance was measured at a wavelength of 490 nm.

To determine the specific antibody titer in antisera, another ELISA was conducted following the aforementioned protocol. In this assay, HPV58 L1VLP or prokaryotic expressed HPV16 L2 aa.11–200 were used as the coating antigens. Following blocking, serially diluted antisera (100 μl/well) were added as the primary antibody. HRP-conjugated goat anti-mouse IgG (1:3000, CWBIO, Cat.: CW0102S) served as the secondary antibody. The antibody titer of a serum is defined as the reciprocal of the highest dilution at which the optical density (OD) value is twice that of the control and exceeds 0.2.

Immunization

BALB/c mice were purchased from the Institute of Laboratory Animal Science (ILAS), Chinese Academy of Medical Sciences (CAMS), and kept in the animal facility under pathogen-free conditions. All animal studies were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee of the ILAS, CAMS with the approval number of ACUC-A02- 2016-001.

Six-week-old female BALB/c mice were randomly distributed into seven groups (five mice per group) and received subcutaneous vaccination at weeks 0, 4, 7, 10 with a dose of 10 μg of 58L1-16L2 cVLPs formulated with Alum-MPL, respectively. The control group was vaccinated with HPV58 L1VLP adjuvanted with Alum-MPL. Serum samples were collected at week 12 for ELISA and pseudovirus-based neutralization assay (PBNA).

HPV pseudovirus preparation

Pseudovirus (PsV) stocks of HPV5, 6, 11, 16, 18, 27, 31, 33, 35, 39, 45, 52, 57, 58, 59, 68 with reporter plasmid pfwB, which encoding green fluorescence protein (GFP) were produced in HEK293 TT cells as described before.[41,42] PsV infectivity (infectious units per ml, IU/mL) was titrated by GFP expression in HEK293 TT cells as described in online technical files (http://home.ccr.cancer.gov/lco/pseudovirusproduction.htm).

Standard PsV-based neutralization assay (PBNA)

Standard PBNA was conducted following established protocols.[41,42] Briefly, HEK293 TT cells were seeded in a 96-

well plate at a density of 3×10^4 cells/well and incubated at 37°C for 6 h. Serially diluted antisera were combined with PsVs and incubated for 1 h at 4°C. The mixtures were then transferred to pre-plated HEK293 TT cells. After 72 h, the cells were harvested by trypsin digestion and analyzed using fluorescence-activated cell sorting. The endpoint titer was determined as the reciprocal of the highest serum dilution resulting in more than 50% inhibition of infection, and this determination was confirmed by two independent replicates.

Data analysis

Statistical analyses were conducted with GraphPad cameyo software with two-tailed, unpaired *t*-test analysis. p values < 0.05 was considered significant.

Results

Design and expression of chimeric 58L1-16L2 constructs

An optimized *N*- and C-terminal truncated HPV58 L1 gene, previously reported by our lab,[39] was used as the backbone for inserting HPV16 L2 RG1 peptide genes into either the h4 coil region or the DE loop (Table 1). Seven chimeric 58L1-16L2 genes were generated, including four chimeras with DE loop insertion and three with h4 coil insertion. Their expression in the baculovirus expression system was confirmed through SDS-PAGE (Figure 1(a)) and Western blot analysis with mAb Camvir-1 (Figure 1(b)). As expected, the 58L1-16L2 chimeric proteins showed a single band at approximately 55 kDa (Figure 1(a,b)), consistent with their theoretical molecular weights, and exhibited a slightly slower migration compared to the HPV58 L1 control.

Purification of chimeric 58L1-16L2 proteins

Chimeric 58L1-16L2 proteins were purified by ultracentrifugation and further analyzed by SDS-PAGE (Figure 2(a)) and quantified with Bradford assay (Figure 2(b)). Bands running at approximately 55 kDa on SDS-PAGE were observed when 5 μg of chimeric proteins were loaded (Figure 2(a)). Their yields varied among 28.9 mg/L–54.4 mg/L, which was about 48%–90.5% of that of HPV58 L1 (Figure 2(b)). Western blot analysis showed that these proteins had a very similar reactivity profile with Camvir-1 and HPV16 L2 antisera (Figure 2(c, d)). It is notable that the four chimeric proteins with DE loop insertions also showed smaller bands representing proteolytic degradation products, indicating that these insertions of L2 peptides might partially interfere with protein stability, but the degraded proteins still retained HPV16 L2 peptides. The remaining three proteins with h4 coil insertions showed non-inferior stability compared to the HPV58 L1 control.

Characterization of chimeric 58L1-16L2 proteins

DLS analysis of seven purified 58L1-16L2 proteins revealed a uniform hydrodynamic diameter distribution, with the mean polydispersity indexes (PDI) of less than 0.2. However, we found that the average hydrodynamic diameters of the four

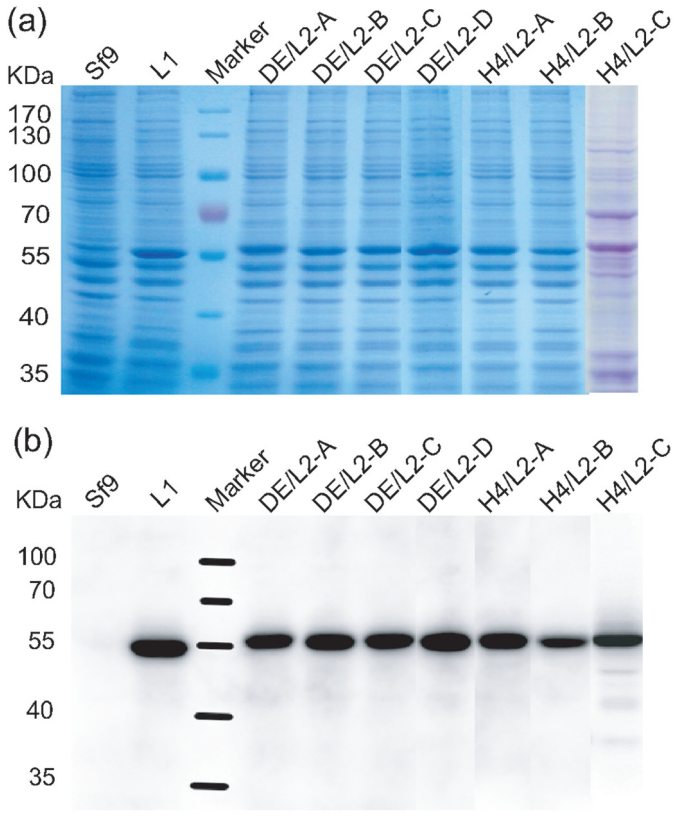

Figure 1. SDS-PAGE with Coomassie blue staining and Western blot analysis of the expression of chimeric 58L1-16L2 genes in sf9 cells. Lysates of sf9 cells expressing seven chimeric 58L1-16L2 proteins or HPV58 L1 control were analyzed by SDS-PAGE with Coomassie blue staining (a), and Western blot with mAb Camvir-1 reacting HPV58 L1 (b).

DE loop-chimeric proteins (that is 92.4 nm, 90.6 nm, 155.6 nm, 73.9 nm) were somewhat larger than those of three h4 coil-chimeric proteins (that is 35.8 nm, 45.2 nm, and 65.02 nm) (Figure 3(a)). Transmission electron microscopy (TEM) analysis showed that the chimeric proteins were all successfully assembled into VLPs, with the average diameters of 60 nm, 50 nm, 45 nm, 50 nm, 30 nm, 35 nm, and 48 nm, respectively (Figure 3(b)), consistent with the results of DLS. These findings suggested that the insertions of L2 peptides had some impact on VLP assembly. It was reported that the insertion of HPV33 or HPV58 L2 peptide into the h4 coil region of HPV18 L1VLP or insertion of HPV31 RG1 peptide into the h4 coil region of HPV16 L1VLP, all resulted in smaller sized VLPs (diameter, 20–40 nm) while had no impact on L1-specific immunogenicity.[21,23] Thus, we speculate that the H4/L2-A, H4/L2-B, and H4/L2-C cVLPs may also provide similar immunogenicity as regular sized VLPs.

Detection of L1 and L2 epitopes displayed on the 58L1-16L2 cVLPs

The presentation of L1 and L2 epitopes on the cVLPs was evaluated via ELISA. As shown in Figure 4(a), the seven cVLPs reacted with HPV16 L2 antisera with varying degrees while HPV58 L1VLP and PBS control did not, indicating the successfully display of L2 epitope peptides on the surface of these cVLPs. The strongest binding was observed for H4/L2-A

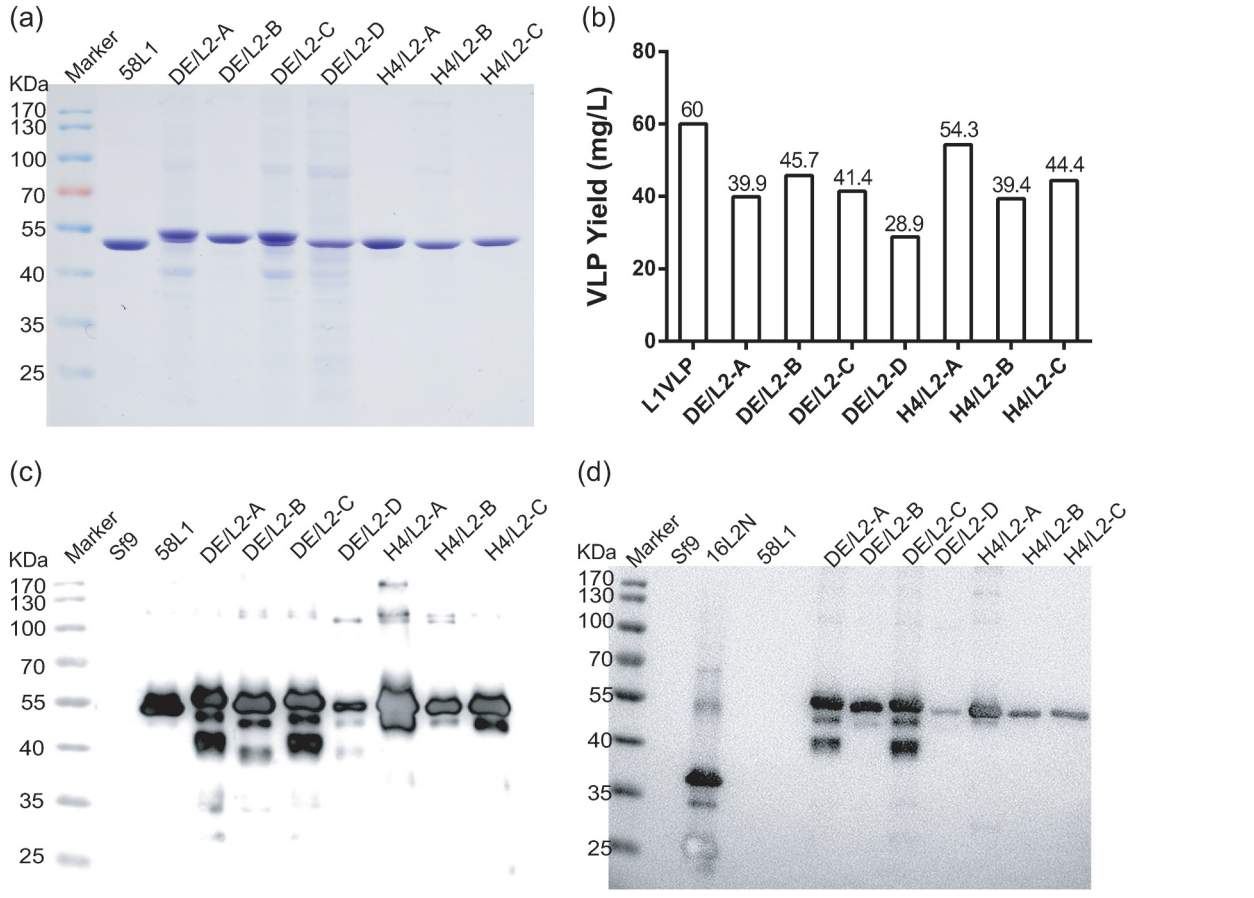

Figure 2. Purity, yield and antigenicity analysis of the chimeric 58L1-16L2 proteins after purification. Chimeric proteins were purified by ultracentrifugation, and the purity was analyzed by SDS-PAGE with Coomassie blue staining (a). The resulting yields were qualified by Bradford assay and recorded as milligrams per liter of medium (b). The antigenicity of these proteins were analyzed by Western blot with Camvir-1 (c) or polyclonal antiserum of KLH-HPV16 L2aa.17–40 (d). 16L2N: HPV16 L2aa.11–200 coupled with the FliC protein of E. *coli*.

and H4/L2-B cVLPs. Besides, one of the conformation-dependent neutralizing mAbs, XM58–6, bound to all the cVLPs, while the remaining five mAbs showed a vary pattern of interaction: no binding to DE/L2-B, DE/L2-C, and DE/L2-D cVLPs; diminished affinity for DE/L2-A cVLP; and the strongest binding affinities with H4/L2-A, H4/L2-B, and H4/L2-C cVLPs (Figure 4(b)). This observation indicated that the insertions of the L2 peptides in the DE loop may result the disruption or steric hindrance of the epitope(s) targeted by these mAbs. Also, the possibility of protein degradation cannot be excluded.

The immunogenicity of 58L1-16L2 cVLPs

Immunogenicity of 58L1-16L2 cVLPs and humoral immune responses to displayed L2 peptides were determined by immunization of BALB/c mice. Concretely, mice were injected with 10 μg per dose of each antigen plus human-applicable Alum-MPL adjuvant at weeks 0, 4, 7, 10. And at week 12, sera were pooled and analyzed by VLP or L2 peptide-based ELISA and standard PBNA. As shown in Figure 5(a,b) three cVLPs, H4/L2-A, H4/L2-B, and H4/L2-C, induced robust HPV58-specific IgG and neutralizing antibody (nAb) titers (10^6), which were comparable to those induced by HPV58 L1VLP, indicating that the major HPV58-specific binding and neutralization epitopes were retained on these cVLPs. However, the antibody titers elicited by the four cVLPs with DE loop insertions (DE/L2-A, DE/L2-B, DE/L2-C, and DE/L2-D) were two orders of magnitude lower ($p < .001$), indicating the destruction of specific neutralization epitope (s) on these cVLPs, which is consistent with the results of Figure 4(b). In addition, the specific IgG and nAbs targeting inserted HPV16 L2 epitopes were also detected. As shown in Figure 5(c,d) the seven cVLPs induced diverse levels of HPV16-specific IgG and nAbs, while none were detected in the HPV58 L1VLP control group, suggesting that HPV16-specific antibody response were raised by the displayed HPV16 L2 epitopes on these cVLPs. It is noteworthy that the antibody titers specific to HPV16 elicited by cVLPs with h4 coil insertions were marginally higher, albeit not significantly, compared to those induced by cVLPs with DE loop insertions.

Therefore, we further detected the cross-neutralizing activity of antisera elicited by H4/L2-A, H4/L2-B, and H4/L2-C cVLPs. Altogether, another 14 HPV PsVs accessible to us were employed in PBNA for determining the cross-neutralizing antibodies in the cVLP antisera. Antibody titers below detection limit (25) were defined as ns. As shown in Figure 6, the antisera of H4/L2-A, H4/L2-B, and H4/L2-C cross-neutralized all detected PsV types with varying efficacy, including high-risk type HPV18, 31, 33, 35, 39, 45, 52, 59, 68 (titer, 25–800), low-

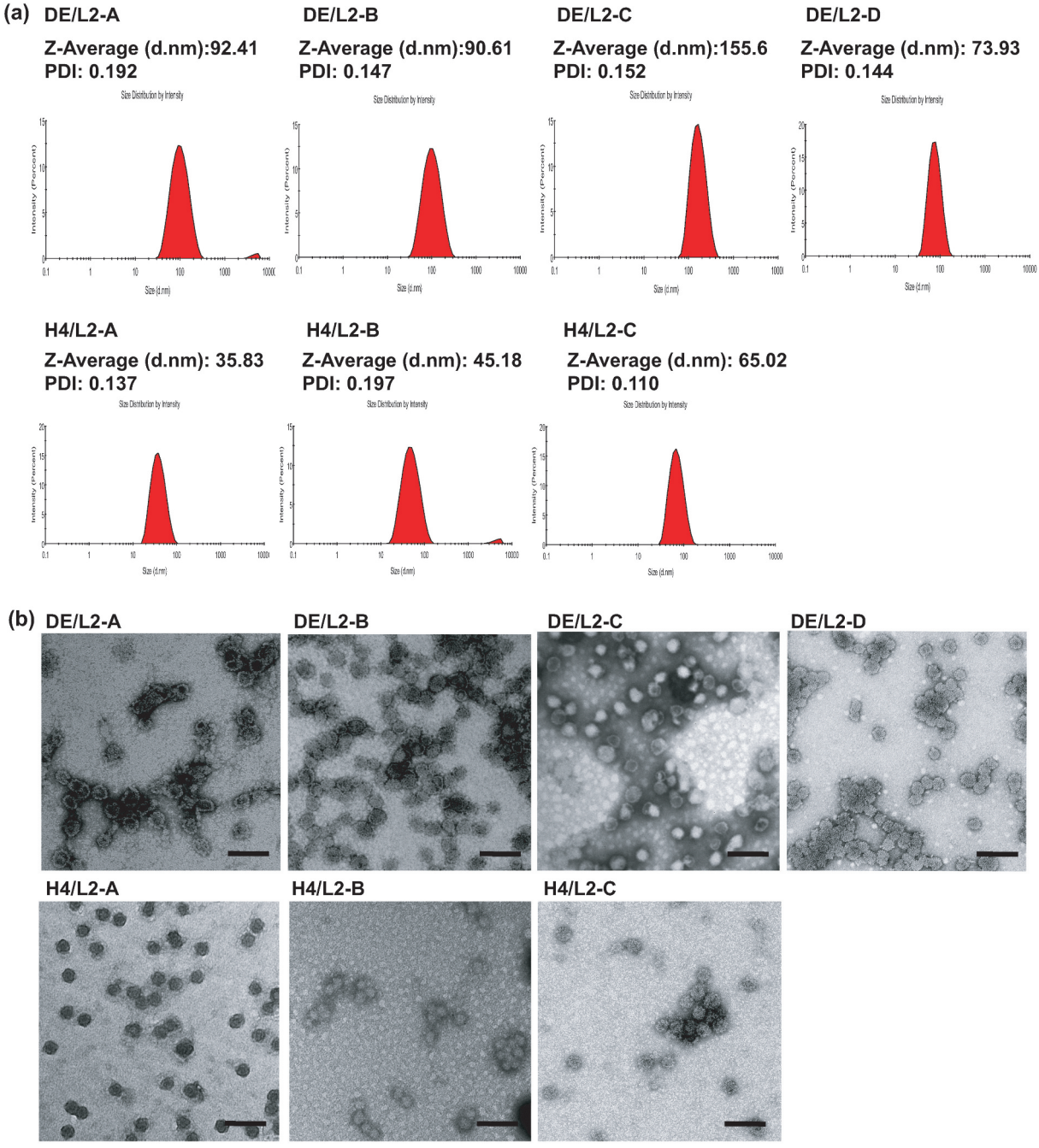

Figure 3. DLS and TEM analysis of the chimeric 58L1-16L2 proteins. Chimeric proteins purified by ultracentrifugation were analyzed by DLS (a) and TEM (80,000× magnification) (b). The Z-average diameter and particle dispersion index (PDI) are also shown. Scale bar = 100 nm.

risk type 6,11 (titer, 25–200), and cutaneous type HPV5, 27, 57 (titer 25–800). The antibody level in antisera raised against H4/L2-A cVLP that displayed the long HPV16 L2 epitope peptide was marginally higher than that of the other two cVLPs with the relatively short L2 epitope peptides, suggesting that the h4 coil region of HPV58 L1 may serve as a potential location for RG1 epitope presentation and can accommodate peptide insertions of at least 21 amino acids in length.

Discussion

In addition to HPV16 and 18, HPV58 is the third most common oncogenic type in Asia, with high detection rate of 7.4% in cervical cancer and 7.7% in high-grade cervical lesions.[37] At present, only one licensed vaccine Gardasil-9 provides protection specifically against HPV58. It has been proven that a broad-spectrum L1-L2 cVLP vaccine could effectively induce long-lasting L1-specific protection as well as L2-specific cross-protection,[21,22,24] thus would be a promising candidate of cost-effective broad-spectrum vaccine. For instance, cVLP vaccines derived from HPV16 and 18 L1VLP have been documented, incorporating either the DE loop or h4 coil chimeric L2 epitopes.[21,22,24] Specific location for the chimeric insertion in HPV58 remains unknown. To our knowledge, we believe this is the first report of HPV58 L1VLP-based L1-L2 chimeric vaccine.

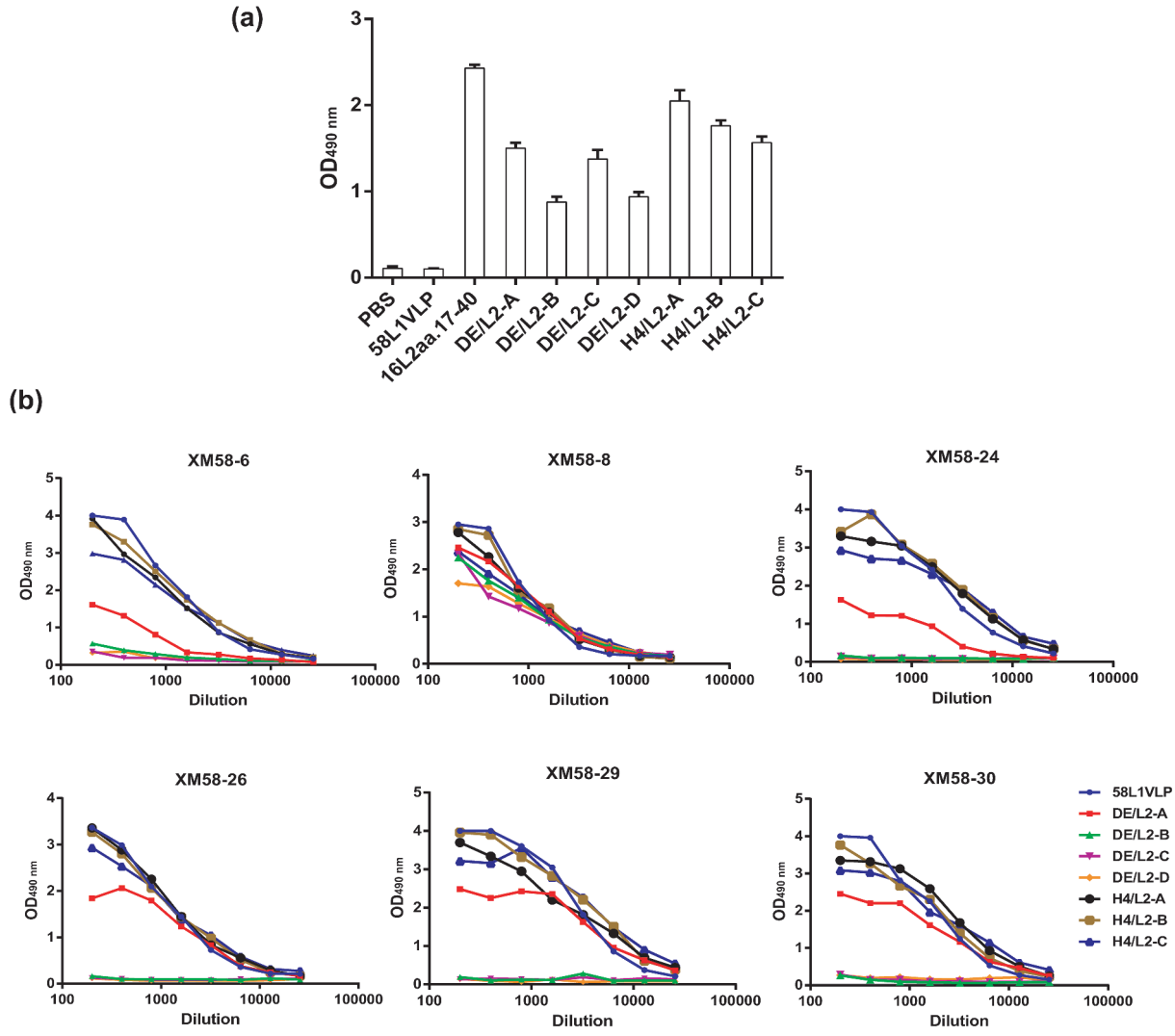

Figure 4. Characterization of L1 and L2 specific epitopes displayed on the cVLPs by ELISA. Binding activities of polyclonal and monoclonal antibodies to 58L1-16L2 cVLPs and HPV58 L1 VLPs were analyzed using polyclonal anti-L2 serum (a) and conformational neutralizing mAbs XM58–6, XM58–8, XM58–24, XM58–26, XM58–29, Xm58–30 produced by our lab (b).

Here, we used a HPV58 L1 mutant which showed enhanced VLP yield in the insect baculovirus expression system as the backbone for L2 epitope insertion,[39] to ensure the yields of the resulting cVLP vaccines and thus their cost-effectiveness. Fortunately, the selected cVLP, H4/L2-A, exhibited a remarkable yield of up to 54 mg/L comparable to that of HPV58 L1, thereby holds significant research value for a promising candidate of a cost-effective broad-spectrum vaccine. Since the position of the L2 epitope display is critical for maintaining L1-specific neutralizing epitopes, both DE loop and h4 coil region of this HPV58 L1 backbone were tried for insertion with type-common RG1 epitope-derived peptides (either long peptides or short peptides) to explore proper positions for display of the epitopes. We found that the chimeras with h4 coil insertions not only retained the L1-specific epitopes but also effectively displayed L2 epitopes on the surface, inducing cross-neutralization of fourteen tested HPV PsVs in vitro, including nine high-risk types, two low-risk types, and three cutaneous types. These results suggest that the h4 coil region of HPV58 L1 might potentially be a good choice for incorporating RG1 epitope peptides to construct chimeric proteins, facilitating the development of broad-spectrum multivalent vaccines based on HPV58.

Although the studies by Huber *et al.* and Carter *et al.* suggested that the DE loops of HPV16 and 18 may encompass crucial sequences associated with dominant neutralizing epitopes,[25,43] successful cases have been reported for the construction of cVLPs using HPV16 and 18 L1 as backbones, with epitopes embedded in the DE loop.[22–24] However, in this study, all the four constructs with DE loop insertions have partially lost the ability to induce HPV58-specific immune responses in mice. This reduction in immunogenicity is likely attributed to the disruption or alteration of epitope(s) targeted by HPV58-specific neutralizing mAbs due to the insertion of L2 peptide sequences or some flanking amino acid modifications. Our speculation is further supported by the findings of He *et al.* that some residues on DE loop of HPV58 L1 interacted with neutralizing monoclonal antibody 5G9 and maybe the key residues involved in the formation of neutralizing epitopes.[44,45] Thus, when inserting epitopes into DE loop of HPV58 L1 to

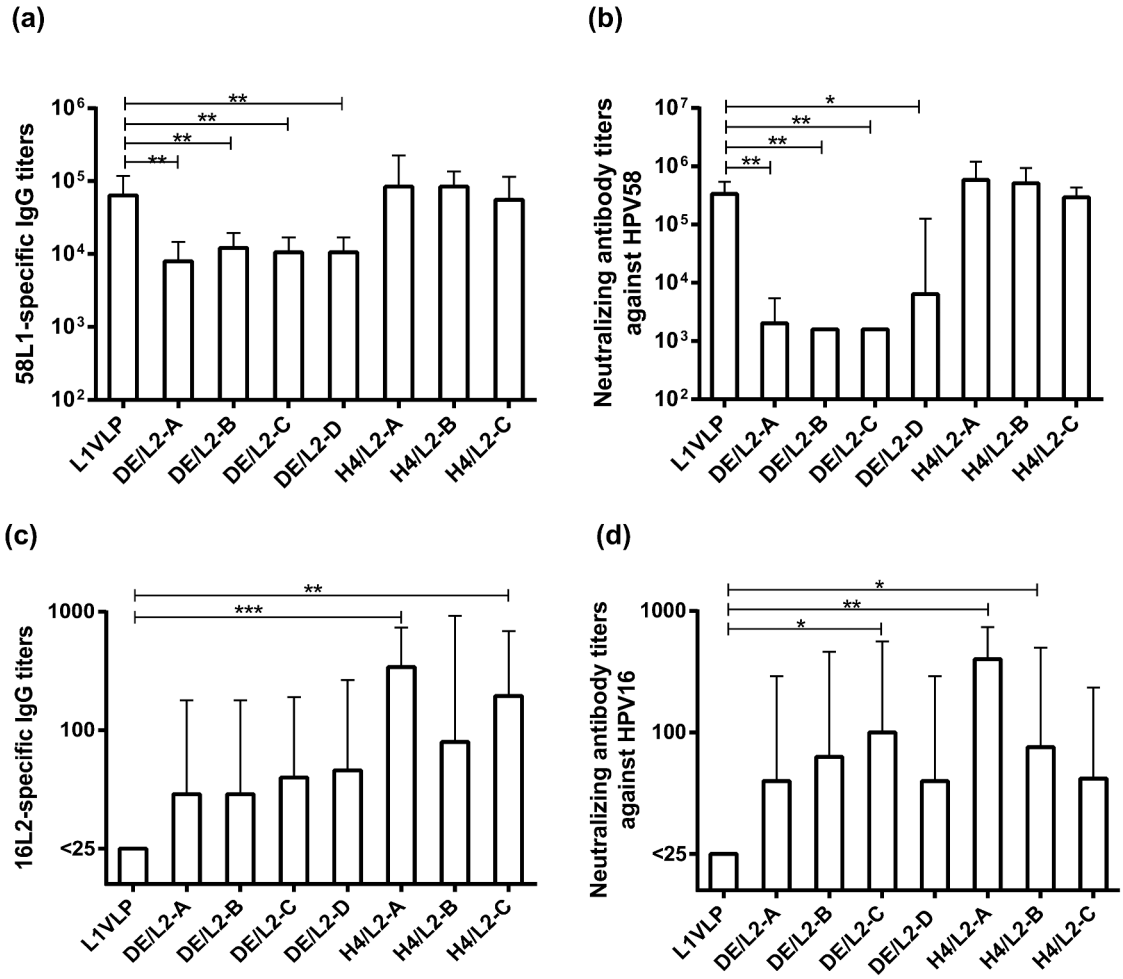

Figure 5. HPV58 and HPV16 specific IgG and neutralizing antibody titers in mice sera raised against 58L1-16L2 cVLPs. Mice ($n = 5$) were vaccinated subcutaneously at weeks 0, 4, 7, 10 with HPV58 L1VLP or 58L1-16L2 cVLPs adjuvanted with Alum-MPL. Sera were collected at week 12 and analyzed for specific IgG titers and neutralizing antibody titers against HPV58 (a, b) or HPV16 (c, d). Data are presented as geometric mean titers (GMTs) with 95% CI. The statistically significant differences were indicated by: *p < .05; **p < .001; ***p < .0001.

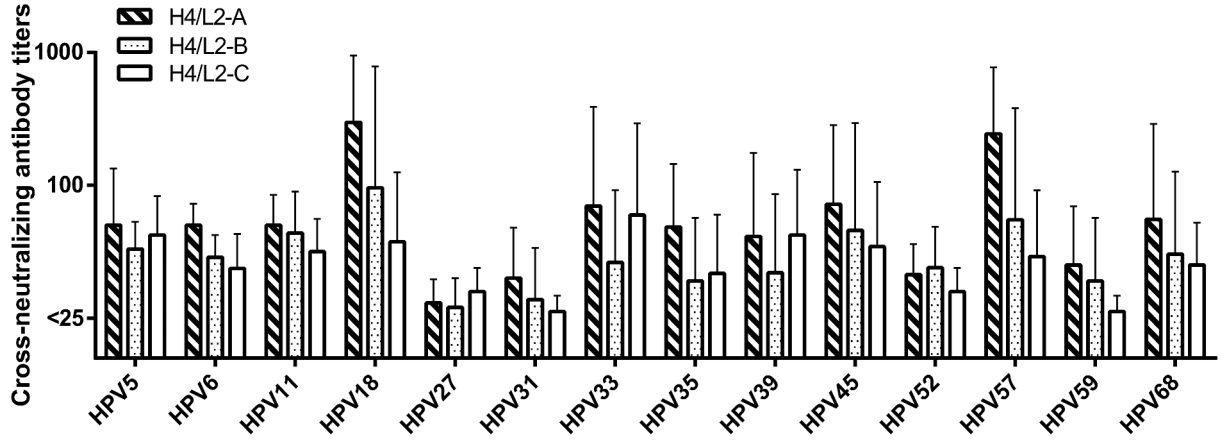

Figure 6. Cross-neutralizing antibody titers in the sera of mice vaccinated with H4/L2-A, H4/L2-B and H4/L2-C cVLPs. Mice ($n = 5$) were vaccinated subcutaneously at weeks 0, 4, 7, 10 with H4/L2-A, H4/L2-B and H4/L2-C cVLPs adjuvanted with Alum-MPL. Sera were collected at week 12 and analyzed for cross-neutralization against other fourteen HPV PsV types. Data are presented as geometric mean titers (GMTs) with 95% CI.

construct cVLPs, it is essential to choose the insertion site and epitope sequence carefully without destroying the L1-specific epitope(s). This might be the reason why we failed to obtain a good immunogenic cVLP with the DE loop insertions in our limited attempts.

As is known to all, the HPV16 L2 cross-neutralization B cell epitope RG1 is highly conserved among many PV types and had been used for broad-spectrum vaccines by multimeric presentation with different scaffolds.[46 – 47] While it seems that the cross-neutralizing antibody responses (mode or

intensity) induced by RG1 peptide varied when delivered with different carrier proteins or immunized with different kind of animals.[24,46,47] For example, 16RG1 peptide displayed on DE loop of BPV1 L1 and HPV16 L1 (16L1-RG1 and BPV-RG1) could induce L2-specific cross-neutralization antibody responses in rabbits, but the titers induced by 16L1-RG1 were 10- to 100-fold higher than those of sera raised against the comparable BPV-RG1 under identical vaccination conditions. Besides, BPV-RG1 could induce cross-neutralizing antibody against low-risk types HPV6, 11 and β genus HPV5 in rabbits, but not in mice.[24,47] In this study, the chimeric 58L1-16L2 cVLPs adjuvanted with Alum-MPL induced cross-neutralization of high-risk HPV types 16, 18, 31, 33, 35, 39, 45, 52, 59, and 68, as well as low-risk types 6 and 11, and beta HPV type 5 in mice. We believed that the RG1 epitope was displayed within the h4 coil of HPV58 L1VLP. It is worth noting, however, that the RG1 epitope-specific antibody titers observed in our study were slightly lower than those reported for successful L1-L2 cVLPs in the literature, which utilized a comparable vaccination strategy, animal model, and detection method.[21,22,48] A potential reason for this difference, as we speculate, could be the significant variation in the presentation mode of the RG1 epitope, which depends on the underlying L1 scaffold sequence. To enhance the vaccine efficacy, further optimization might be necessary, such as optimizing the structure of the chimeric protein by carefully adjusting the linker amino acids around the insertion site[50] to ensure better exposure of the L2 epitopes. Additionally, exploring alternative novel adjuvants or modifying the existing Alum-MPL adjuvant formulation could help boost the antigen-specific immune response.[51,52] Furthermore, we postulated that a more potent and broader immune response might be elicited in rabbit models by our 58L1-16L2 cVLPs, as compared to the response observed in mice.[22,24]

Regarding the weak immunogenicity of the linear L2 epitope, L2-targeted vaccine formulations usually require potent immune adjuvants and multiple booster immunizations to enhance the levels of specific neutralizing antibodies in vivo.[22–25–52] In this study, a four-dose immunization regimen was adopted, which aligns with established cVLP vaccine protocols.[21–24] Given that the antibody levels below the detection threshold of in vitro L1-based PBNA can still protect animals from the in vivo PsV challenge,[53] it is imperative in future studies to conduct an extended longitudinal monitoring of antibody levels throughout the immunization schedule for comprehensive evaluation of the vaccine's efficacy.

The L1 protein can self-assemble into 50 to 60 nm VLPs with a $T = 7$ icosahedral symmetry, which is crucial for its potent immune activity. While other higher-order structures also exhibit immunogenicity, such as smaller $T = 1$ VLPs (30–40 nm in diameter), which also induced comparable antibody titers to those elicited by full-sized $T = 7$ VLPs.[54] Similarly, the H4/L2-A cVLP generated in this study also possessed a particle diameter of around 30 nm, suggesting it might be a $T = 1$ icosahedral symmetry, and as expected, induced a non-inferior specific humoral immune response to that induced by the HPV58 L1VLP ($T = 7$, 55 nm). It is consistent with the previous findings on the chimeric constructs with h4 coil insertions of HPV16 and 18 that formed small VLPs of approximately 30 nm in size and elicited significant immune response.[21,23]

In summary, our results suggested the h4 coil region of HPV58 L1VLP might be a potential location for RG1 epitope display, providing valuable guidance for future research aimed at developing broad-spectrum chimeric vaccines based on HPV58 L1VLP.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

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Author contributions statement

Zhirong Wang performed experiment, acquired data, and drafted the manuscript. Ting Zhang was involved in analysis and interpretation of data. Xuemei Xu made contributions to conception and design, and revised it critically for important intellectual content. All authors read and approved the final manuscript.

Data availability statement

The original contributions presented in the study are included in the article; further inquiries can be directed to the corresponding author.

Ethical statement

No ethical approval was required as the study did not involve human participants or identifiable personal data.

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