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A rationally designed flagellin-L2 fusion protein induced serum and mucosal neutralizing antibodies against multiple HPV types

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ABSTRACT

The amino terminus of human papillomavirus (HPV) minor capsid protein L2 harbors several conserved neutralizing epitopes, including aa.17–36 (RG-1 epitope) and aa.65–85 consensus epitope (cL2 epitope), which are considered to be promising for the construction of cost-effective pan-HPV vaccine candidates. However, the immunogenicity of L2 epitope/peptide is rather weak, and the neutralizing spectrum induced by single type of L2 antigen is suboptimal. In this study, we constructed L2 concatemer with HPV18/33/58/59 RG-1 epitopes and 16L2 aa.11–88 peptide, and fused it with flagellin, a strong systemic and mucosal adjuvant, by hypervariable region replacement. A copy of cL2 epitope was also introduced to the C-terminus of the recombinant protein. The resultant Fla-5PcL2 protein can be produced in *E. coli* expression system with high yield and good stability. We assessed the immunogenicity of Fla-5PcL2 in mouse model via systemic and mucosal route, and found that subcutaneous immunization with Fla-5PcL2 induced robust serum neutralizing antibodies against divergent HPV types, while intranasal immunization with Fla-5PcL2 induced remarkable L2-specific IgA and cross-neutralizing antibodies in mucosal secretions, and medium titers of cross-neutralizing antibodies in sera. Moreover, Fla-5PcL2 induced full protection against vaginal HPV challenges. As mucosal antibodies provide the first-line defense at infection sites, and needle-free immunizations may increase vaccine compliance and require less public health resources, our results demonstrate that Fla-5PcL2 is a promising vaccine candidate which possibly meet the need in low-resource regions.

1. Introduction

Over 200 human papillomavirus (HPV) types have been identified and classified into five genera (α, β, γ, μ, η) according to L1 gene sequences [1,2]. Persistent infection with mucosal oncogenic HPVs, of which more than 20 types were identified (HPV 16, -18, -26, -31, -33, -35, -39, -45, -51, -52, -53, -56, -58, -59, -66, -67, -68, -70, -73, -82, *etc.*) [3], is associated with nearly all cervical cancers and a great portion of other anogenital (vaginal, vulvar, penile and anal) and oropharyngeal cancers [4,5], altogether account for nearly 4.8% of global cancer burden. Mucosal low-risk HPVs (HPV6, -11, *etc.*) are linked to benign genital warts and recurrent respiratory papillomatosis. Cutaneous HPVs can be found in all 5 genera, some of which, such as HPV2, -27, -57 (α4), are associated with benign common and plantar warts [6]; other types, such as HPV5, -8 (β1), are related to the development of non-melanoma skin cancers in epidermodysplasia verruciformis patients [6].

All of the currently licensed HPV vaccines (bivalent Cervarix, quadrivalent Gardasil and nonavalent Gardasil 9) are L1 virus-like particle (VLP)-based vaccines, which induce mainly type-specific neutralizing antibody responses and provide limited cross-protection against closely related non-vaccine types [7–9]. The complexity of manufacture and requirement of cooling chains during delivery are the principal impediment to a broad implementation of HPV vaccines in developing regions, where nearly 90% of cervical cancer deaths occur [10,11]. Moreover, adding more types of VLPs to broaden the vaccine spectrum is likely to further drive up the vaccine complexity and thus increase the cost. Therefore, the development of cost-effective pan-HPV vaccine is highly desired.

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Immunization with minor capsid protein L2 or its N-terminal peptides induced cross-neutralizing antibodies and protection in animal models [12,13]. Several conserved neutralizing epitopes have been identified in L2 N-terminus [14–16], among which the RG-1 epitope (HPV16 L2 aa. 17–36) is considered to be a major cross-neutralizing epitope [17–19], and the consensus epitope of L2 aa.65–85 (cL2) is also proved to induce cross-neutralizing antibodies when chemically conjugated to bacteriophage capsid [20]. However, the immunogenicity of L2 peptide/epitope is intrinsically weak and the neutralizing spectrum induced by a single type of L2 antigen is suboptimal [21,22]. Many efforts have been made to enhance the immunogenicity and extend the protection spectrum of L2-based vaccines, including formulating L2 concatemers with potent adjuvants [21,23], displaying L2 epitopes on the surface of VLPs [24–31], and delivering multimeric L2 epitopes/peptides with monomeric protein scaffold, such as FcγR-targeting scaffolds [32,33], bacterial thioredoxin (Trx) [22,34,35] or flagellin scaffold [17,36].

Flagellin, the major component of bacterial flagella and the agonist of toll-like receptor 5 (TLR5) [37], exhibits strong systemic and mucosal adjuvant properties when administered with different antigens [37–40], especially in covalently linked form [41,42]. As a bacterial structure protein, flagellin can be expressed at a high level with good solubility in prokaryotic expression systems, which makes it an ideal displaying scaffold for epitopes/peptides with multiple cysteines (*e.g.* RG-1 epitopes) [17,43–45]. The FliC flagellin of *Salmonella enterica* serovar Typhimurium can be structurally divided into 4 domains, named D0, D1, D2 and D3 [46,47]. The D0 and D1 domains are highly conserved and essential for TLR5 signaling, while the D2 and D3 are hypervariable with the major intrinsic antigenic determinants [47,48]. Partial or complete deletion of the hypervariable regions of FliC significantly reduced its antigenicity but preserved adjuvanticity [48–50]. Previous study on flagellin-based influenza vaccines revealed that fusing HA antigens at both the D3 domain and C-terminus of flagellin resulted in higher levels of neutralizing antibodies and protective responses comparing with fusing the antigen at either the D3 domain or C-terminus [51]. Pioneer works on flagellin-L2 vaccines demonstrated that intramuscularly immunization with the recombinant proteins, constructed by fusing L2 concatemers at the C-terminus of D3 domain-deleted flagellin, induced durable protection against cutaneous challenge with diverse HPV quasivirions [17,36], thus suggested a possible way to develop broad-spectrum vaccine based on single antigen.

Mucosal immune responses provide the first-line defense at infection sites. Induction of mucosal neutralizing antibodies may provide optimum protection of mucosal surfaces against HPV infections. However, mucosal antibodies are poorly induced by systemic immunizations [52,53]. Intranasal immunization with flagellin fusion antigens can induce specific mucosal immune responses at oral, respiratory tract, and genital-vaginal mucosa [54–60], thus making flagellin-L2 fusion proteins attractive for HPV mucosal vaccine development.

In this study, we constructed L2 concatemers with rationally selected RG-1 epitopes according to their immunogenicity, and fused the L2 concatemers to flagellin by replacement of D2 and D3 domains. A cL2 epitope was also introduced at the C-terminus of flagellin. Subcutaneous immunization with the resultant Fla-5PcL2 fusion protein induced cross-neutralizing antibodies against divergent HPV types. Intranasal immunization with Fla-5PcL2 induced cross-neutralizing antibodies in both sera and mucosal secretions. Moreover, Fla-5PcL2 induced potent protection against vaginal pseudovirus (PsV) challenges when administered either subcutaneously or intranasally.

2. Materials and methods

2.1. Construction, expression and purification of recombinant Fla-L2 proteins

The L2 concatemers 5PL2 and 7PL2 were constructed with five or seven types of L2 peptides respectively as illustrated in Fig. 1A, and GS linkers were inserted at each of the type-to-type junctions. The amino acid sequence of the consensus epitope of L2 aa.65–85 (cL2) was GTGGRTGYVPLGTRPPTVVDV, which was described previously [20]. 5PL2, 7PL2 and cL2 genes (Gene Bank accession: MK896848, MK896849 and MK896850, respectively) were codon optimized according to the codon usage bias of *E. coli* and synthesized (Sangon, Shanghai, China). FliC gene (Gene Bank accession: AAL20871) was amplified by PCR from genomic DNA of *Salmonella typhimurium LT2*. The D2 and D3 domain-deleted flagellin construct was generated by replacement of D2 and D3 region (aa.177–401) with a multicloning site linker GGGGSASLKGGGGS. Fla-5PL2 and Fla-7PL2 were constructed by inserting of 5PL2 or 7PL2 genes into the multicloning site respectively, and Fla-5PcL2 was constructed by fusing cL2 epitope to the C-terminus of Fla-5PL2 via PCR. An octahistidine tag was added at the C-terminus of each construct. The recombinant genes were cloned into pET22b plasmid (Novagen, USA) for fusion protein expression.

To express the fusion proteins, plasmids were transformed into *E. coli* BL21 (DE3) (Novagen, USA), and bacteria were incubated in auto-inducing media ZYM-5052 [61] at 37 °C for 20–24 h. The *E. coli* cells were collected and resuspended in PBS, and then subjected to ultrasonication. After centrifuged at 12,000 rpm for 10 min, the supernatant and pellet were collected for analysis. Fla-5PL2, Fla-5PcL2, Fla-7PL2 and 7PL2 proteins were purified from inclusion body by Ni-Sepharose affinity chromatography according to the manufacturer's instructions (GE Healthcare, USA). After purification, proteins were renatured by two-step dialysis at 4 °C with Renature Buffer (1 M Urea, 50 mM Tris, 150 mM NaCl, 3 mM Glutathione, 1 mM Oxiglutathione, pH 8.0) for 24 h and then PBS for 24 h. Endotoxin was removed by 1% Trixton X-114 partitioning as described elsewhere [62]. Protein expression and purity was analyzed by SDS-PAGE with Coomassie blue staining. Antigenicity of L2 peptides was confirmed by Western blot with anti-HPV16 L2 polyclonal antibodies. The amount of residual endotoxin in purified proteins was determined by Tachypleus Amebocyte Lysate (TAL) assay (Chinese Horseshoe Crab Reagent Manufactory, China). Endotoxin levels were below 2 EU/mg. The hydrodynamic size of recombinant Fla-L2 protein was recorded as Z-average hydrodynamic diameter detected by dynamic light scattering (DLS) analysis performed on a Malvern Zetasizer Nano ZS (Malvern, USA) at room temperature.

2.2. Enzyme linked immunosorbent assay

The binding of recombinant proteins to L2 polyclonal antibodies was analyzed by ELISA as described before [33]. Briefly, ELISA plates were coated with serial dilutions of Fla-5PL2, Fla-5PcL2, Fla-7PL2 and 7PL2 (0.2–100 ng/well) at 4 °C overnight. After blocking, anti-HPV 16L2, 18L2, 31L2 or 6L2 polyclonal antibodies (diluted at 1:3000) was respectively added to the plates and incubated at RT for 2 h, followed by incubating with HRP-conjugated goat anti-rabbit IgG (1:3000) at 37 °C for 1 h. Enzyme activity was revealed with substrate O-phenylenediamine (Sigma-Aldrich, Missouri, USA) and the reaction was stopped by 2 M H_2SO_4. The absorbance was measured at 490 nm. The anti-L2 polyclonal antibodies were produced by immunization with RG-1 epitopes of

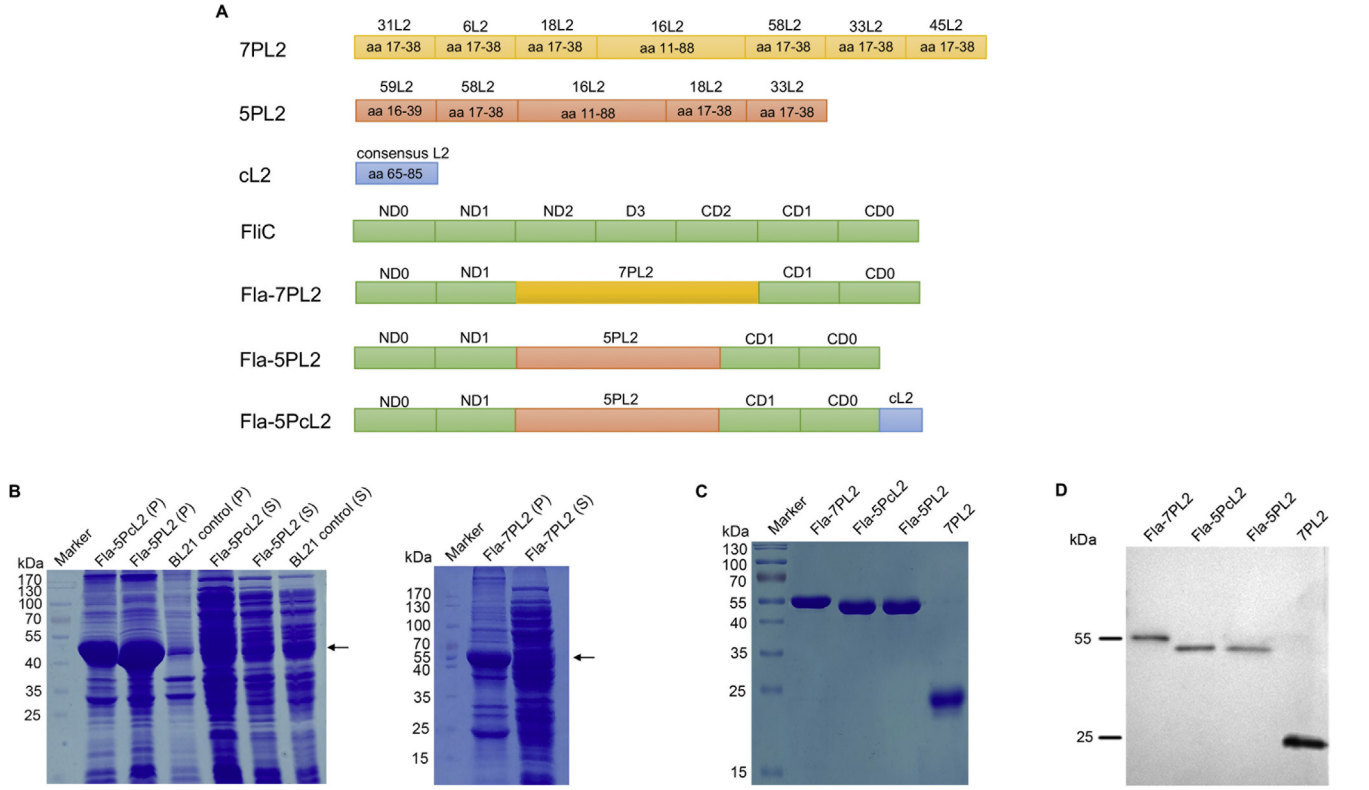

Fig. 1. Expression and purification of recombinant proteins. (A) Schematic diagram of Fla-L2 recombinant proteins. cL2, consensus L2. (B) The recombinant Fla-L2 proteins were expressed in BL21 (DE3), and the expression levels were performed by SDS-PAGE with Coomassie blue staining. P, pellet; S, supernatant. (C) The purity of recombinant proteins was also examined by SDS-PAGE with Coomassie blue staining. 10 μg of each recombinant protein was loaded. (D) The insertion of L2 peptides was analyzed with Western blot using anti-16L2 rabbit polyclonal antibodies (1:2000).

each type respectively. Specifically, New Zealand white rabbits were subcutaneously immunized with 0.5 mg KLH-conjugated RG-1 epitopes formulated with Freud's Adjuvant at day 0, 14, 28, 42, 56. The immune serum was collected at day 72 and incubated at 56 °C for 30 min prior to use.

The L2-specific antibody titers of sera, saliva and vaginal washes were also analyzed by ELISA. Briefly, ELISA plates were coated with 7PL2 peptides (100 ng/well) respectively at 4 °C overnight. After blocking, plates were incubated with 2-fold serially diluted (start at 1:5) immune sera, saliva or vaginal washes at RT for 2 h, and then detected by HRP-conjugated rabbit anti-mouse IgA (1:1000), HRP-conjugated rabbit anti-mouse IgG1 (1:1000) or HRP-conjugated rabbit anti-mouse IgG2a (1:1000). Enzyme activity was measured as above. The end point titers were calculated as the reciprocal of the highest dilution with an absorbance greater than 0.1 and 2-fold higher than control serum/saliva/vaginal wash at the same dilution.

2.3. Animal immunization

BALB/c mice were purchased from Institute of Laboratory Animal Science, Chinese Academy of Medical Sciences (CAMS) and kept in the animal facility of the Institute of Basic Medical Sciences (IBMS), CAMS, under pathogen-free conditions. All animal studies were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee (IACUC) of IBMS, CAMS, and all protocols were reviewed and approved by IACUC (Approval Number: NCC2016-005).

Four- to six-week-old female BALB/c mice were randomly distributed into groups of 5, and were vaccinated subcutaneously for four times at two-week intervals with 25 μg of Fla-5PcL2, Fla-7PL2, 7PL2 peptide or flagellin respectively. For adjuvant evaluation, 25 μg of Fla-5PcL2 was formulated with either 50 μg Aluminum hydroxide adjuvant (Alum), or 50 μg Alum and 5 μg MPL (Alum/MPL), and then mice were immunized subcutaneously for four times at two-week intervals. For mucosal immunity analysis, mice were anesthetized and immunized intranasally with 25 μg of Fla-5PcL2 or flagellin in 15 μl PBS for four times at two-week intervals. Two weeks after the last immunization, sera, saliva and vaginal washes were collected and subjected to pseudovirus-based neutralization assay. Serum samples were collected from the tail vein. Vaginal washes were collected on 5 consecutive days by flushing the mouse's vagina with 0.1 ml of PBS. Saliva samples were collected as described before [63]. Briefly, mice were anesthetized and injected intraperitoneally with 10 μl/g body weight pilocarpine, and transfer the saliva into collection tube once the saliva is observed in mouth.

2.4. Standard PsV-based neutralization assay (PBNA)

BPV1 and HPV2, -5, -6, -11, -16, -18, -27, -31, -33, -35, -39, -45, -52, -57, -58, -59, -68 PsVs were produced and titrated as described previously [32,33,64–67], and then used for standard PBNA as described elsewhere [33,64–66]. Briefly, 293TT cells were seeded in 96-well plate at 1.5×10^4 cells/well and incubated overnight at 37 °C. The sera were 2-fold serially diluted in 96-well plate (start at 1:5), and mixed with equal volume of PsV diluent. The mixture of serially diluted sera and PsVs were transferred to pre-plated 293TT cells and cultured for 72 h. The 293TT cells were harvested and detected by FACS. The endpoint titer was calculated as the reciprocal of the highest serum dilution with percent infection inhibition higher than 50%. The neutralizing activity was consid-

ered to be undetectable when the percent infection inhibition was lower than 50% at the lowest serum dilution (1:5). The mean values and SD are reported.

2.5. Murine vaginal PsV challenge

Murine vaginal HPV PsV challenge was performed as described before [25,68]. Briefly, mice were treated with 3 mg of progesterone subcutaneously 4 days before PsV challenge, and were intravaginally pretreated with 50 μl of 4% nonoxynol-9 (Sigma-Aldrich, USA) 6 h prior to PsV challenge. After that, 20 μl of PsV preparation containing about 5×10^5 IU of PsVs (encapsidated reporter plasmid pLucf) and 1% CMC (Sigma-Aldrich, USA) was intravaginally instilled. Forty-eight hours post challenge, mice were vaginally instilled with 0.4 mg of 5′-F-Luciferin (CellCyto Life Sciences, China). Three minutes later, luciferase signals were acquired for 10 min with an IVIS 200 bioluminescent imaging system (Xenogen, USA).

2.6. Data analysis

Statistical significance was determined by Student's *t*-test and one-way ANOVA with Bonferroni's multiple test correction using SPSS software.

3. Results

3.1. Preparation and characterization of Fla-L2 proteins

As illustrated in Fig. 1A, 7PL2 peptide was composed of 16L2 aa.11–88 and RG-1 epitopes from 6 HPV types (HPV18, -33, -45, -31, -58/-52, -6) selected according to HPV prevalence [69]. Previous studies reported that the immunogenicity of 6RG-1 and 45RG-1 epitopes was very weak [25,28], and thus induced low titers of cross-neutralizing antibodies even in the context of VLP [28]. So, we constructed another L2 concatemer containing 16L2 peptide and 4 potent RG-1 epitopes from HPV18, -33, -58, and -59. Both 7PL2 and 5PL2 were fused to flagellin by D2D3 domain replacement strategy, as the fusion proteins constructed with this strategy possibly have better immunogenicity [56,70].

The expression of Fla-5PL2, Fla-5PcL2 and Fla-7PL2 in BL21 (DE3) was efficiently induced after cultured in ZYM-5052 media for 20–24 h, and the recombinant proteins were mainly expressed in inclusion bodies (Fig. 1B). The recombinant Fla-L2 and 7PL2 proteins were purified by Ni-Sepharose affinity chromatography. The purity was examined by SDS-PAGE and Coomassie blue staining, and a single band was observed when 10 μg of recombinant protein was loaded (Fig. 1C). All expressed proteins were confirmed by Western blot with anti-HPV16 L2 polyclonal antibodies (Fig. 1D).

As flagellins tend to polymerize in solution, we detected the hydrodynamic diameters of Fla-L2 proteins by DLS to evaluate their polymeric level, and observed that Fla-5PL2 was highly polymerized with a diameter of 4911 nm, while the polymeric levels of Fla-5PcL2 and Fla-7PL2 were remarkably lower (Table 1 and Fig. S1B). Moreover, we also noticed that the refolding rate and yield of Fla-5PcL2 and Fla-7PL2 were higher than that of Fla-5PL2. The yield of Fla-5PcL2 in lab scale reached as high as 89 mg/L, which was the highest among the three Fla-L2 proteins.

The exposure of inserted L2 peptides in Fla-L2 proteins was evaluated by ELISA (Fig. S2). ELISA plates were coated with 2-fold serial dilutions of Fla-5PL2, Fla-5PcL2, Fla-7PL2 and 7PL2 (start at 100 ng/well) and detected by polyclonal antibodies against RG-1 epitopes. Polyclonal antibodies against HPV16, -18, -31, and -6 RG-1 epitopes bound strongly to Fla-5PcL2 and Fla-7PL2, suggesting that L2 peptides in Fla-5PcL2 and Fla-7PL2 were well exposed. However, the binding activity of Fla-5PL2 was lower than that of Fla-5PcL2 and Fla-7PL2 even at high coating concentrations, indicating that the aggregation of Fla-5PL2 strongly influenced the exposure of inserted L2 peptide.

Table 1
Expression, yield and characteristics of recombinant Fla-L2 proteins.

	Fla-5PL2	Fla-5PcL2	Fla-7PL2
Expression	Inclusion bodies	Inclusion bodies	Inclusion bodies
Refolding method	Two-step dialysis	Two-step dialysis	Two-step dialysis
Refolding rate	21%	92%	75%
Yield (mg/L)	16.5	89	60.5
Polymeric level	High	Low	Low
Size ± St Dev (d. nm)[a]	4911 ± 785.1	11.83 ± 1.88	22.73 ± 6.58
Isoelectric point (pI)[b]	7.49	8.05	5.31

[a] The hydrodynamic size of recombinant Fla-L2 protein was recorded as Z-average hydrodynamic diameter detected by DLS analysis.
[b] The isoelectric points were calculated according to the amino acid sequences of recombinant proteins with DNAMAN X software (Lynnon Biosoft, Version 10.002).

3.2. Fla-5PcL2 and Fla-7PL2 induced cross-neutralizing antibodies against divergent HPV types

As Fla-5PL2 is easy to aggregate and unstable for storage (Fig. S1C), we only use two Fla-L2 fusion proteins (Fla-5PcL2 and Fla-7PL2) for immunogenicity evaluation. We immunized mice subcutaneously with Fla-5PcL2, Fla-7PL2, 7PL2 and flagellin respectively. Two weeks after the 4th immunization, the immune sera were collected and determined by standard PBNA. Both Fla-5PcL2 and Fla-7PL2 induced cross-neutralizing antibodies against all 17 tested HPV types (Fig. 2), and the titers were significantly higher than that induced by 7PL2 peptide ($P < 0.01$), indicating immunogenicity enhancement by flagellin scaffold. Fla-5PcL2 induced robust titer of HPV16 neutralizing antibodies (mean titer, 3486), which is significantly higher than that induced by Fla-7PL2 ($P < 0.01$). Moreover, medium titers of neutralizing antibodies were detected against HPV31, -59, -2, -39, -18, -33, -45, and -58 (mean titers ranged from 135 to 432), among which the titers against HPV58, -59 and -2 were significantly higher than that of Fla-7PL2 ($P < 0.05$, $P < 0.01$). Low titers of neutralizing antibodies against HPV52, -57, -6, -5, -68, -11, -35 and -27 (mean titer ranged from 16 to 98) were also observed. The neutralizing antibodies were not detected in flagellin control group. The results suggested that the immunogenicity of Fla-5PcL2 was higher than Fla-7PL2. To rule out the interference from non-specific reactions, we also detected the neutralizing activities of the immune sera against BPV PsVs, and found that the neutralizing activity was undetectable even at a dilution of 1:5.

3.3. Formulation with Alum and MPL cannot enhance the humoral immunity induced by Fla-5PcL2

In order to elucidate whether the addition of adjuvants further enhance the immunogenicity of Fla-L2 proteins, we formulated Fla-5PcL2 with Alum or Alum/MPL respectively. After 4 subcutaneous immunizations, mice sera were evaluated by standard PBNA and L2-based ELISA. To our surprise, the neutralizing antibody titers against HPV16, -18 and -52 were not enhanced by either Alum or Alum/MPL ($P > 0.05$, Fig. 3A), and the titers against HPV58 were almost the same when immunized with or without Alum adjuvant ($P > 0.05$, Fig. 3A), suggesting that addition of Alum and/or MPL have little impact on neutralizing antibody titers

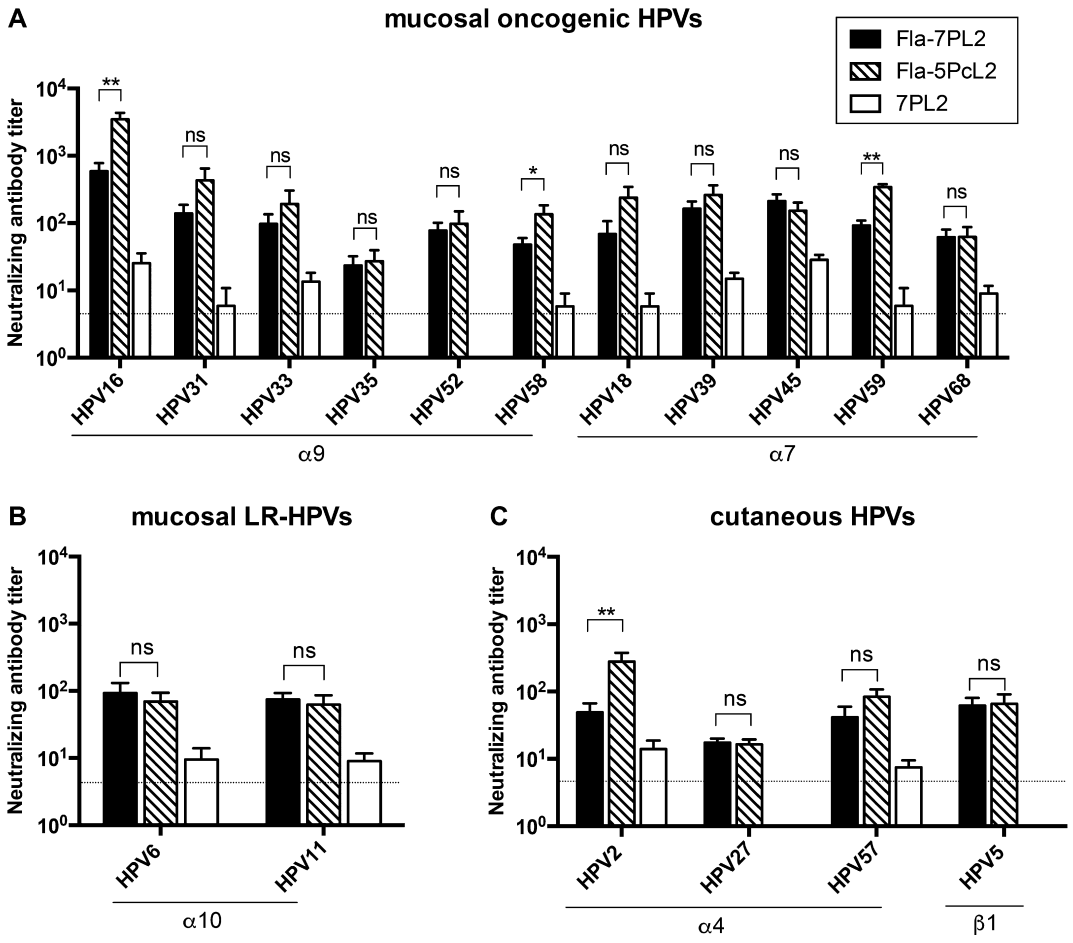

Fig. 2. Broad-spectrum neutralizing antibody titers induced by Fla-L2 proteins. Groups of mice (n = 5) were immunized subcutaneously in weeks 0, 2, 4 and 6 with 25 μg Fla-L2 proteins or 7PL2 peptide. Sera were collected at week 8 and tested for in vitro neutralizing antibody titers against mucosal oncogenic HPVs (A), mucosal LR-HPVs (B) and cutaneous HPVs (C). The results were presented as mean titer ± SD, and the dotted line indicates the limit of detection for the assay. The statistically significant differences (using one-way ANOVA) were indicated by: *, $P < 0.05$; **, $P < 0.01$; ns, $P > 0.05$.

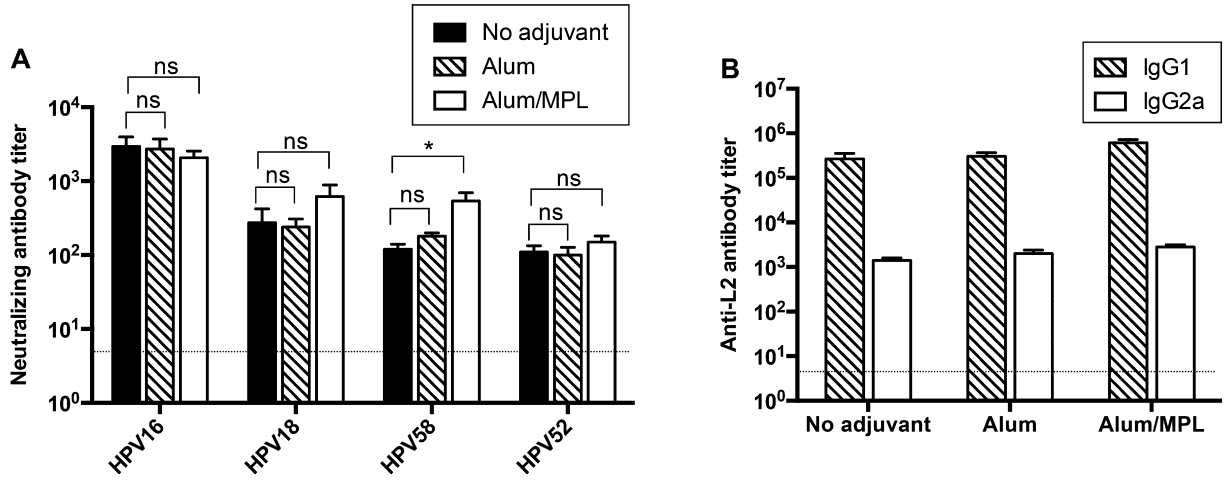

Fig. 3. Neutralizing antibody and sera IgG responses to Fla-5PcL2 with or without adjuvants. Groups of mice (n = 5) were immunized subcutaneously in weeks 0, 2, 4 and 6 with Fla-5PcL2 protein (25 μg) alone or formulated with Alum (50 μg), or Alum (50 μg) plus MPL (5 μg). Sera were collected at week 8 and tested for in vitro neutralizing antibody titers (A) and anti-L2 IgG1/IgG2a titers (B). ELISA plates were coated with 7PL2 peptides (100 ng/well), and binding with serially diluted sera was detected with HRP-conjugated rabbit-anti-mouse IgG1 or HRP-conjugated rabbit-anti-mouse IgG2a. Reactivity was determined by measuring the mean optical density (OD) values at 490 nm. The results were presented as mean titer ± SD, and the dotted line indicates the limit of detection for the assay. The statistically significant differences (using one-way ANOVA) were indicated by: *, $P < 0.05$; ns, $P > 0.05$.

induced by Flagllin-L2 fusion proteins in mice. We further compared the L2-specific IgG1 and IgG2a titers between groups, and observed that the IgG1 and IgG2a titers of no adjuvant group were similar to those of the two adjuvanted groups ($P > 0.05$, Fig. 3B), indicating that the Th1/Th2 bias was not altered by the addition of adjuvants, although flagellin usually induce Th2 biased immune responses while MPL tend to tune the immunity toward Th1 biased responses. We can conclude that the flagellin is a potent self-adjuvant and additional adjuvants are not necessary.

3.4. Mucosal immunization with Fla-5PcL2 induced serum and mucosal IgA and neutralizing antibodies

Until present, no successful HPV mucosal vaccine have been reported. To investigate whether flagellin-L2 fusion proteins could be applied as mucosal vaccines, we immunized groups of mice (n = 5) intranasally with Fla-5PcL2, and determined the sera and mucosal antibody responses by standard PBNA and L2-based ELISA. As illustrated in Fig. 4A, low to medium levels of cross-neutralizing antibodies against HPV16, -18, -33, -58, -31 (mean titers ranged from 26 to 104) were detected in the immune sera, and the titers were only about 1 order of magnitude lower than those induced by systemic immunization. Moreover, we also observed detectable levels of neutralizing antibodies against all tested HPV types in saliva and vaginal washes. Next, we determined the L2-specific IgA titers and found that the intranasally immunized mice showed IgA titers of 51, 33 and 5 in sera, saliva and vaginal washes respectively (Fig. 4B). The results demonstrate that Fla-5PcL2 is a potential mucosal vaccine candidate, and could induce sufficient mucosal and medium systemic immune responses when administered intranasally. The saliva and vaginal washes of flagellin control group and systemically immunized mice were also evaluated, but the neutralizing antibodies and IgA titers were all undetectable. Previous studies showed that systemic immunization with HPV L1 VLPs or capsomeres could induce detectable levels of specific IgG/IgA or neutralizing antibodies in mucosal secretions, but the titers were usually 2–4 orders of magnitude lower than detected in immune sera [71–73]. As the highest sera neutralization titer induced by Fla-L2 subcutaneous immunization was $\sim 10^3$, we speculate that the neutralizing antibody levels in the mucosal secretions were below the detection threshold.

3.5. Subcutaneous and mucosal immunization with Fla-5PcL2 induced protection against vaginal HPV PsV challenge

As the antibody titer threshold for protection has not been clearly identified, we further analyzed the protective efficacy induced by Fla-5PcL2. Two weeks after the last immunization, mice were vaginally challenged with HPV39, -58 and -5 PsVs. The results suggested that both subcutaneous and mucosal immunization with Fla-5PcL2 induced full protection against HPV39, -58 and -5 PsVs (Fig. 5), indicating that detectable levels of neutralizing antibodies are sufficient for protection against HPV PsV challenge in mice.

4. Discussion

As the spectrum of neutralizing antibodies induced by single type of RG-1 epitope is suboptimal, multiple RG-1 epitopes from divergent HPVs would be included in a pan-HPV vaccine. However, RG-1 epitope contains two conserved cysteines (homologues to C22 and C28 of HPV16 L2), which limit the copy number of RG-1 epitopes delivering within single protein scaffold. Previous studies revealed that 3 copies of RG-1 epitopes in tandem displayed by FcR targeting scaffolds [32,33] or Trx [22,35] showed optimal stability and immunogenicity, while including 6–12 copies of RG-1 epitopes in tandem resulted in high degradation rate and low expression level [32,33], possibly due to the unexpected disulfide cross-linking between epitopes. In this study, we arranged 2–3 copies of RG-1 epitopes as structure units, and fused them to both N- and C-terminus of HPV16 L2 aa.11–88 peptide to construct L2 concatemers 5PL2 and 7PL2. Both 5PL2 and 7PL2 can be expressed stably in *E. coli* system (the expression data of 5PL2 were not shown). Non-reducing PAGE analysis revealed that the intermolecular disulfide cross-linking levels of Fla-5PL2, Fla-5PcL2 and Fla-7PL2 were similarly low (Fig. S1A), but the hydrodynamic diameter of Fla-L2 proteins varied (Fig. S1B), suggesting that the different levels of polymerization were possibly driven by non-covalent interactions. We noticed that the isoelectric point

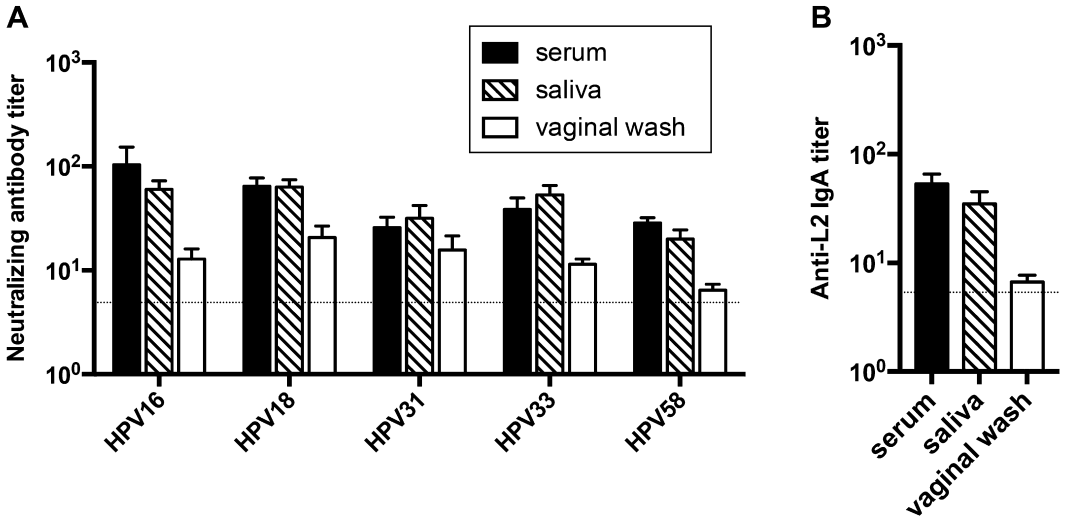

Fig. 4. Neutralizing antibody and vaginal IgA responses to Fla-5PcL2 intranasal immunization. Groups of mice (n = 5) were immunized intranasally in weeks 0, 2, 4 and 6 with 25 μg Fla-5PcL2 protein. Sera, saliva and vaginal washes were collected at week 8 and tested for in vitro neutralizing antibody titers (A) and anti-L2 IgA titiers (B). ELISA plates were coated with 7PL2 peptides (100 ng/well), and binding with serially diluted sera, saliva or vaginal washes were detected with HRP-conjugated rabbit-anti-mouse IgA. Reactivity was determined by measuring the mean optical density (OD) values at 490 nm. The results were presented as mean titer ± SD, and the dotted line indicates the limit of detection for the assay.

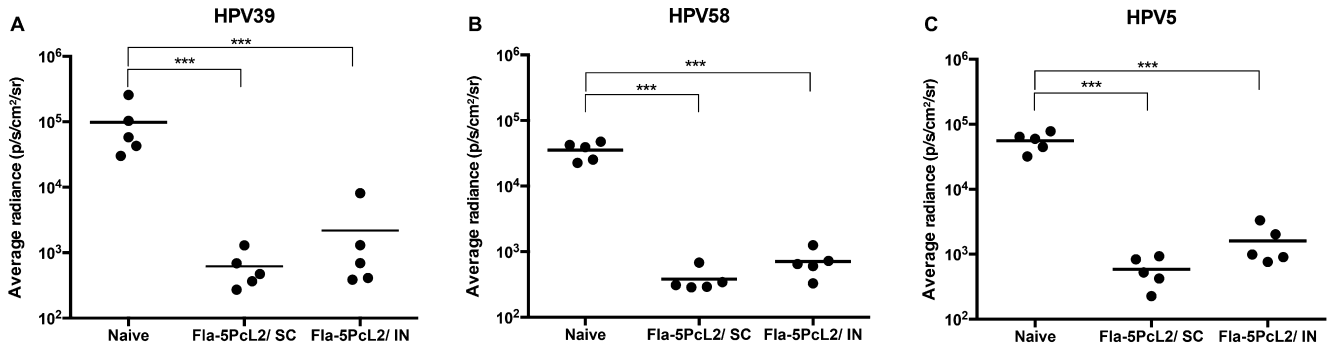

Fig. 5. Protective immunity induced by Fla-5PcL2. Groups of mice (n = 5) were immunized subcutaneously or intranasally in weeks 0, 2, 4 and 6 with 25 μg Fla-5PcL2 protein. At week 8, mice were challenged with HPV39 PsVs (A), HPV58 PsVs (B) and HPV5 PsVs (C). The statistically significant differences (using one-way ANOVA) were indicated by: ***, $P < 0.001$.

(pI) of Fla-5PL2 is 7.49 (Table 1), suggesting low solubility and tendency to aggregate at neutral pH. Fusion with cL2 epitope solved the problem as the pI of Fla-5PcL2 increased to 8.05 (Table 1). We also observed that Fla-5PcL2 can be kept stably at RT for at least 10 days with less than 10% aggregation (Fig. S1C), while a major portion of Fla-7PL2 aggregated within 2 days. As the cL2 epitope is mainly composed of polar amino acids, we speculate that introducing cL2 epitope at the C-terminus may facilitate the stability of Fla-L2 proteins in solution.

Structural and functional analysis of flagellin demonstrate that TLR5 recognizes flagellin monomers but not filaments as the TLR5 recognition site is shielded after flagellum assembly [47]. Another study showed that nanoparticles formed by flagellin-HA fusion proteins with hydrodynamic diameters of 47.7–81.7 nm exhibited similar levels of TLR5 agonist activity as FliC, and induced full protection against lethal H3N2 virus challenge [74], indicating that oligomerization of flagellin fusion proteins would not impair the adjuvant activity. The hydrodynamic diameters of intact Fla-5PcL2 and Fla-7PL2 were 11.83 nm and 22.73 nm respectively (Table 1), but the diameters of denatured Fla-L2 were about 1 nm, suggesting that Fla-5PcL2 and Fla-7PL2 existed in oligomeric form in solution. Both Fla-5PL2 and Fla-7PL2 induced significantly higher levels of cross-neutralizing antibodies than L2 concatemer 7PL2 (Fig. 2), also proved that the adjuvant activity of flagellin was preserved.

Pioneer studies on HPV L2-flagellin fusion proteins showed that systemic immunization with L2-flagellin fusion proteins (Fla69/Fla ~5 × 11–88, Fla65 and Fla76) induced protection against HPV6, -16, -18, -31 and -58 [17,36], but neutralizing antibody and protective immunity induced by mucosal immunization route was not analyzed. The L2 concatemers used in previous studies were composed of L2 fragments from HPV6, -16, -18, -31, -39 and/or -52, and were fused to the C-terminus of D3-domain-deleted flagellin. In our study, 7PL2 was composed of 16L2 aa.11–88 and RG-1 epitopes from most prevalent HPV types (HPV18, -33, -45, -31, -58/-52 and -6), and 5PL2 was composed of 16L2 aa.11–88 and highly immunogenic RG-1 epitopes (HPV18, -33, -58/-52 and -59), RG-1 epitopes with low immunogenicity (such as 6RG-1 [25] and 45RG-1 [28]) were ruled out. Previous reports showed that hypervariable region replacement seems to be a good strategy for construction of flagellin fusion proteins with Influenza HA [51,75], HIV-1 p24 [56], or *S. mutans* PAC [70], as the resultant fusion proteins induced more potent protective immune responses than their counterparts constructed by C-terminal fusion strategy. So, in this study, 7PL2 and 5PL2 were respectively fused to flagellin by hypervariable region (D2 and D3) replacement. Our results showed that, the L2 concatemers were well exposed on the surface of Fla-7PL2 and Fla-5PcL2 (Fig. S2), and the two fusion proteins induced similar levels of broad-spectrum neutralizing antibodies (Fig. 2), indicating that increasing the copy number of RG-1 epitopes does not necessarily improve the immunogenicity. Prediction data by homology modeling showed that, when fused with flagellin, 7PL2 presented a more complicated structure than 5PL2 (Fig. S3), which probably reduced the exposure of some RG-1 epitopes. Thus, we speculate that broader spectrum and higher immunogenicity of L2 vaccines is more likely to be achieved by using limited copies of potent L2 epitopes rather than including as many epitopes as possible. Moreover, we also observed that intranasal immunization of Fla-5PcL2 fusion protein induced neutralizing antibody responses and protective immunity, indicating the possibility to apply Fla-5PcL2 fusion protein as needle-free vaccine, which may require less technical support and reduce the cost of immunization.

In conclusion, we demonstrate that Fla-5PcL2, constructed by fusing L2 peptides at both the hypervariable region and C-terminus of flagellin, induced robust broad-spectrum serum neutralizing antibodies via systemic immunization, elicited remarkable mucosal neutralizing antibodies via intranasal immunization, and also induced protection against vaginal PsV challenges via both routes. Besides, Fla-5PcL2 can be produced in *E. coli* system with high yield and good stability. Thus, the strategy we reported here may provide a reference for the development of L2-based pan-HPV vaccines.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vaccine.2019.06.002.

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