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Development of prophylactic recombinant HPV58-attenuated *Shigella* live vector vaccine and evaluation of its protective efficacy and immunogenicity in the guinea pig keratoconjunctivitis model

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To develop a prophylactic recombinant HPV58L1-attenuated *Shigella* live vector vaccine and evaluate its protective efficacy and immunogenicity in the guinea pig keratoconjunctivitis model, the HPV58L1 gene was cloned into vector pUCmt, and then subcloned into the suicide vector pCVD442. The recombinant plasmid pCVD442-HPV58L1 was introduced into attenuated *Shigella* (sf301:ΔvirG) with the helper plasmid PRK2013 by filter mating. The positive colonies were harvested and confirmed by polymerase chain reaction. The expression of the HPV58L1 protein with a molecular weight of 60 kDa was confirmed by western blot. The ability of the interested protein to self-assemble into virus-like particles was identified by transmission electron microscope, and murine erythrocyte hemagglutination assay. The guinea pig keratoconjunctivitis model was used to evaluate the protective efficacy and immunogenicity of the vaccine. Animal experiments showed that there was no keratoconjunctivitis occurred in the immunized group (HPV58-attenuated *Shigella*), and the serum levels of anti-HPV58L1-IgG and -IgA were obviously increased ($P < 0.05$), but the anti-sf301 LPS-IgG just slightly increased ($P > 0.05$). Enzyme-linked immunosorbent spot assay showed that HPV58L1-specific IgA-antibody-secreting cells (ASC) and IgG-ASC of spleen and lymph nodes were also obviously increased ($P < 0.01$). In this study, a recombinant HPV58L1-attenuated *Shigella* live vector vaccine was successfully constructed, and it could induce strong humoral immune responses in the immunized animals, and induce protective antibody production.

Keywords HPV58; *Shigella*; live vector vaccine; recombination

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Introduction

It has been well accepted that some human cancers are caused by biological agents, such as human papilloma virus (HPV), hepatitis B virus (HBV), Epstein–Barr virus (EBV), and human T-lymphotropic virus I (HTLV). Prevention of the infections by such infect agents is believed to be an essential step to eliminate these cancers. High-risk HPVs are the major initiator of human cervical cancer [1,2], and etiologically linked to almost 100% of cervical cancers. The recognition of a strong etiological relationship between infections of high-risk HPVs and cervical cancer has prompted developing prophylactic and therapeutic vaccines against such agents. Neither infectious HPV virions are able to be isolated from naturally occurring lesions, nor are grown *in vitro* in cell culture or *in vivo* in animals other than in the human body. However, several different types of HPV16 prophylactic vaccines, including DNA vaccine, subunit vaccine, virus-like particles (VLPs) vaccine, and recombinant live vector vaccine, have been developed in recent years due to significant advances in molecular virology and genetic engineering [3]. Among those vaccines, significant progress has been made in the development of HPV VLP prophylactic vaccine. In 2006, the US Food and Drug Administration (FDA)

approved the first prophylactic quadrivalent human papillomavirus (HPV) types 6/11/16/18 L1 VLP vaccine [4]. However, the VLP vaccine is expensive, which makes it unaffordable for the general public, especially those who are living in developing countries. Therefore, researches have been carried out in an effort to develop cheap and effective prophylactic vaccines against the oncogenic types of HPV.

Compared with conventional expression systems, the recombinant live vector vaccine offers many potential advantages, such as the ability to elicit systemic and mucosal immunity, simplicity, convenience, and low cost; therefore, it was considered as a desirable prophylactic HPV vaccine. Among recombinant live vector vaccines, most common vectors were vaccinia virus and bacillus Calmette–Guerin. Recent evidence indicates that using attenuated *Shigella* as live vector has its advantages: weak immunogenicity, unable to elicit permanent immunity, and its infection does not spread to the whole body [5,6]. Hence, attenuated *Shigella* was often used as a vector to prepare recombinant live vector vaccine.

Up to now, more than 100 types of HPV have been characterized. Among them, HPV16 is the most common high-risk type worldwide, and the second one is HPV18. Yet in China, instead of HPV18, HPV58 ranks as the second, associated with cervical cancer [7]. Unfortunately, there are few researches dedicated to HPV58, and no study has been carried out in the development of prophylactic HPV58 vaccine. In this study, we tried to construct the prophylactic recombinant HPV58-attenuated *Shigella* live vector vaccine and evaluate its immunogenicity and protective efficacy in the guinea pig keratoconjunctivitis model.

Materials and Methods

Plasmid construction

It was reported that deletion of C-terminus 34 amino acid of HPV16L1 protein did not interfere with the VLP assembling, even might increase yield [8], so two polymerase chain reaction (PCR) primers 5′-CAGGTCGACA TGTCCGTGTGGCGGCCTAGTGAG-3′ (forward primer) and 5′-GGAGAGCTCTTACTTTCGTCCCAAAGGAAA CTGATCTAGATC-3′ (reverse primer) for the amplification of HPV58L1 (C-terminus 33 amino acid deleted) were designed, with *sal*I/*sac*I sites at the 5′ and 3′ ends. HPV58 genome was used as the template. Plasmid containing HPV58 genome was kindly provided by Prof. Weiming Zhao from Shandong University (Jinan, China). HPV58L1 gene cDNA was amplified by PCR with the above two specific primers and the template plasmid. After PCR, the amplified fragment was cloned into pUCmt (Sangon, Shanghai, China), generating the recombinant pUC-L1, which was identified by restriction enzyme digestion, PCR amplification, and sequencing. The HPV58L1 gene was harvested by *sal*I and *sac*I digestion from pUC-L1, and inserted into suicide vector pCVD442 provided by Prof. Jun Yu (Imperial College, London, UK). The recombinant plasmid pCVD442-HPV58L1 was transformed into cc118 competent cells (gift from Prof. Jun Yu).

Construction of recombinant of HPV58L1-attenuated *Shigella*

Plasmid pCVD442-HPV58L1 was introduced into sf301:ΔvirG from cc118 competent cells with helper plasmid PRK2013 by filter mating. *Shigella* sf301 strain was kindly provided by Prof. Jun Yu. sf301:ΔvirG strain was constructed as previously reported [9]. First, 600 μl sf301:ΔvirG strain and 200 μl helper plasmid PRK2013 and 200 μl plasmid pCVD442-HPV58L1 were combined. Then the mixture was incubated for 2 h at 37°C on LB-agarose medium without resistance and smeared on Congo red (CR) LB-agarose plates with ampicillin and rifampin resistance at 37°C overnight. On the next day, a single clone was selected and incubated in LB liquid substrate without selection. After serial dilution, the cultures were inoculated onto LB plates containing 5% sucrose and low salt at 30°C overnight. The presence of the *SacB* gene in pCVD442 would inhibit the growth on 5% sucrose; growth on sucrose was used as positive selection for the loss of the vector pCVD442. Colonies were picked and tested for ampicillin and CR sensitivity. When a single clone could grow on CR plates, a corresponding clone was resistant to ampicillin resistance plates, the colony was considered as a positive colony which was then selected and identified by PCR [10].

SDS–PAGE and western blot analysis

To identify the HPV58L1 protein expression, after positive colonies with rifamycin resistance were incubated for 24 h in LB, the bacterial extracts were separated by 12% SDS–PAGE, transferred to the blotting membrane, and subjected to western blot probed with anti-HPV16L1 monoclonal antibody. Briefly, proteins were resolved by 12% SDS–PAGE and then transferred to a polyvinylidene difluoride membrane. The membrane was blocked by incubation for 1 h at room temperature in 5% non-fat dry milk and 1% bovine serum albumin in Tris-buffered saline (pH 7.5), then incubated with mouse

anti-HPV16L1 monoclonal antibodies (NeoMarkers, Fremont, USA) at 1:1000. Horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G (DaKo, Carpinteria, USA) was used as the secondary antibody, and diaminobenzidine was used for color development.

Analysis of VLPs by transmission electron microscopy

For analysis of VLP assembly by transmission electron microscopy, the HPV58L1 protein expressed in the recombinant attenuated *Shigella* was dialyzed against 1 mM HEPES for 30 min at room temperature, dropped onto carbon-coated copper grids, and then negatively stained with phosphotungstic acid. Specimens were then observed under an H-600 transmission electron microscope (Hitachi, Tokyo, Japan).

Murine hemagglutination assay

Erythrocyte suspension was prepared from the fresh blood of C57BL/6 mice [11]. HPV58L1 (1 mg/ml) diluted in 50 μl PBS was plated in 96-well plates, and then mixed with an equal volume of 1% (*V*/*V*) suspension of erythrocytes in PBS. After incubation for 3 h at 4°C, the plates were visualized and photographed.

Protection test in animals against *Shigella* infections

Six guinea pigs were divided into two groups: immunization group (HPV58L1-attenuated *Shigella*) and PBS control group. In the immunization group, the animals were given HPV58L1-attenuated *Shigella* in the eye at an average dose of 6×10^8 bacteria per eye. In the PBS group, 20 μl PBS was given. Three weeks later, the animals were challenged with sf301 strain at a dose of 6×10^8 CFU per eye. The animals were observed closely for 7 days to see if there were any abnormal behaviors, particularly the development of keratoconjunctivitis. The severity of keratoconjunctivities was rated on the basis of its latent period and appearance: grade 0, no disease or mild irritation and dissipated rapidly; 1, mild conjunctivitis; 2, keratoconjunctivitis with no purulence; and 3, fully developed keratoconjunctivitis with purulence.

Invasion ability of recombinant strain HPV58L1-attenuated *Shigella*

HeLa cell infection assay was performed to test the invasion ability of the recombinant strain HPV58L1-attenuated *Shigella*. In brief, HeLa cell monolayers were incubated in antibiotic-free Dulbecco's minimal essential medium (DMEM) containing 10% fetal calf serum on 35 mm plates in 5% CO_2 at 37°C to half-confluence. Then, 25 μl of mid-logarithmic phase bacteria was overlaid, spun down to adhere the HeLa cells at 1500 rpm for 10 min, and incubated together in humidified 5% CO_2 at 37°C for 30 min. The HeLa cells were then washed six times with DMEM, and treated with DMEM containing 50 μg/ml gentamicin for 90 min to kill the extracellular bacteria. After washing with PBS, the HeLa cells were fixed with 4% formalin and then Giemsa-stained. The number of intracellular bacteria was counted under microscopy.

Guinea pig keratoconjunctiva immunization protocol

Eighteen healthy guinea pigs were divided into three groups: immunization group (HPV58L1-attenuated *Shigella*), PBS group, and sf301 group. Right before immunization, blood was collected from two guinea pigs each group. The immunization group was challenged via the eye with 6×10^8 HPV58L1-attenuated *Shigella* each animal at Days 0, 2, and 3 for three times, the sf301 group was treated with 6×10^8 sf301 strains, and the PBS group was treated with 20 μl of PBS. After immunization, blood samples were collected from three guinea pigs each group at Day 13 and booster immunization was finished at Days 14 and 15. The animals were sacrificed by ulatan injection for following experiments after blood collection at Day 20 after immunization.

Preparing cells for enzyme-linked immunosorbent spot assay

The animals were dissected and spleens, regional lymph nodes [superficial ventral cervical lymph nodes (SVCLN), mandible nodes (MDLN), and mesenteric nodes (MSLN)], and peyer's patches (pp) were harvested. Lymphocytes were obtained by teasing the lymphoid tissues and passing through a sterile sift to remove debris, then washed once in RPMI 1640 medium containing 15 μg/ml gentamicin. Finally, the erythrocytes were cleaned out by the lysing buffer. The generated lymphocytes were washed one more time with RPMI 1640 medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, and 15 μg/ml gentamicin, and the cell concentration was adjusted to 2×10^5 cells/ml.

Enzyme-linked immunosorbent spot assay

The HPV58L1-specific antibody-secreting cells (ASC) response of the animals to immunization and challenge was determined by enzyme-linked immunosorbent spot (ELISPOT) assay [12] with some modifications. In brief, each well of 96-well microtiter plates was coated with 20 μl HPV58L1 VLP overnight at 4°C. After washing once with PBS, the wells were blocked with 5% fetal

calf serum in PBS. Then, lymphocyte suspension was dispensed into each well, and the plates were incubated for 4 h at 37°C in a humidified CO_2 incubator. Thereafter, the plates were washed three times with PBS–Tween, 100 μl of rabbit anti-guinea pig IgG at 1:1000 (DaKo) and IgA at 1:1000 (Bethyl, Montgomery, USA) were added, respectively, and the plates were incubated at 4°C overnight. Later on, 100 μl of goat anti-rabbit IgG conjugated to alkaline phosphatase (Sigma, St Louis, USA) at 1:1000 was added to each well, and the plates were incubated for 2 h at 37°C. Finally, after the plates were washed with PBS–Tween, 100 μl of melted agarose was added to each well. Antigen-specific lymphocytes were visualized as blue spots, counted under a stereomicroscope, and recorded as ASC per 10^5.

ELISA analysis

Each microtiter well was coated with 100 μl HPV58 VLPs [13], and the plates were incubated overnight at 4°C. After washing, the plates were blocked with 5% goat serum for 1 h at 37°C. After washing with PBS–Tween, guinea pig serum serially diluted 2-fold in casin buffer were added to the wells, and the plates were incubated for 2 h at 37°C. After another washing, 50 μl of rabbit anti-guinea pig IgG (1:1000) and IgA (1:1000) was added to each well, respectively, and the plates were incubated for 2 h at 37°C. Afterwards, 100 μl of goat anti-rabbit IgG (1:1000) conjugated to alkaline phosphatase was added to each well, and the plates were washed with PBS–Tween after 2 h incubation at 37°C. Then, 100 μl of the substrate (1 mg/ml of *p*-nitrophenyl phosphate in 1 M diethanolamine buffer, containing 0.5 mM $MgCl_2$) was added to each well, optical density was read at 450 nm. Titers of sera obtained from the animals prior to immunization were used as controls.

Statistical analysis

One-way analysis of variance (ANOVA) was used to assess the results of immunization responses. If $P < 0.05$, it shows significant differences (by ANOVA). Geometric mean serum titers ($\pm$SD) were used to summarize the serum immune response (IgG, IgA). The SPSS14.0 software was used to carry out all statistical analysis.

Results

Construction of recombinant of HPV58L1-attenuated *Shigella*

Plasmid pUC-HPV58L1 was constructed and verified by sequencing. The HPV58L1 gene fragment is about 1.4 kb; furthermore, the results showed two site mutations, 6175 A → G, resulted Gln → Arg and 6233 T → C, but no amino acid change. After digesting plasmid pUC-HPV58L1 with *sal*I/*sac*I, the HPV58L1 gene fragment was inserted into plasmid pCVD442. The recombinant plasmid was verified by PCR and was named as pCVD442-HPV58L1. Plasmid pCVD442-HPV58L1 was introduced into sf301:ΔvirG from cc118 competent cells with helper plasmid PRK2013 by filter mating. After homologous recombination, a positive colony in the sf301: ΔvirG strains contained the HPV58L1 gene was selected and verified by PCR.

SDS–PAGE and western blot assay

After culturing for 24 h, positive culture was harvested, and analyzed by 12% SDS–PAGE, followed by western blot analysis. Because no specific antibody against HPV58L1 protein is available and the 64% homology in amino acid sequence between HPV16L1 and HPV58L1 proteins, HPV16L1 monoclonal antibody was used for western blot analysis. The HPV58L1 protein of 60 kDa was confirmed, and the results proved that we had successfully expressed HPV58L1 protein in HPV58-attenuated *Shigella* (**Fig. 1**).

Electron microscopic analysis

Morphologically, the self-assembled VLPs from recombinant HPV58L1 protein were confirmed by electron microscopy. Hollow spherical particles with 55 nm in diameter were seen in the recombinant HPV58-attenuated *Shigella* using the H-600 transmission electron microscope, identical to the dimensions of HPV58 VLPs reported previously [13], implying that the recombinant

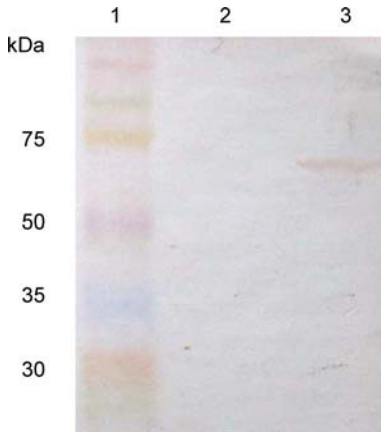

Fig. 1 Western blot analysis of truncated HPV58L1 protein 1, Rainbow marker; 2, sf301:Δvir G strain (negative control); 3, HPV58L1-attenuated *Shigella*.

HPV58L1 protein expressed was able to self-assemble into VLPs (**Fig. 2**).

Biological activity of recombinant HPV58L1 protein

The HPV58L1 protein expressed in recombinant attenuated *Shigella* could cause murine erythrocyte agglutination, but the sf301 and sf301:ΔvirG negtive control under the same conditions did not. The results confirmed that the HPV58L1 aim protein possessed proper bioactivity, and this was simply the necessity of efficacious immune protection (**Fig. 3**).

Immune protection after HPV58-attenuated *Shigella* immunization

Animals immunized with HPV58-attenuated *Shigella* showed no evidence of keratoconjunctivitis, and sf301 challenging also revealed an 83% immune protection. The controls exhibited no any natural resistance against *Shigella* at all. Further, our data showed that the HPV58-attenuated *Shigella* strain was safe as virG virulence gene had been deleted (**Table 1**).

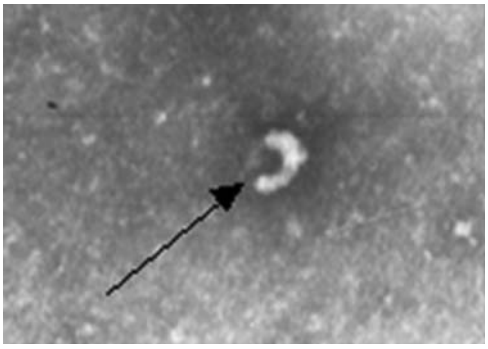

Fig. 2 Electron micrograph of HPV58VLP, 55 nm hollow spherical particles Arrow indicates the VLPs assembled by expressed HPV58L1 protein. Magnification, ×50,000. VLP, virus-like particle.

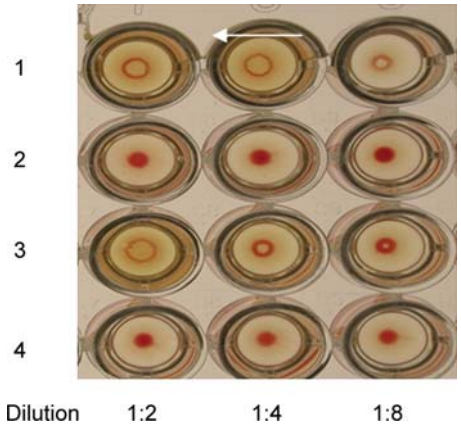

Fig. 3 Result of murine hemagglutination assay of the expressed HPV58L1 protein 1 and 3 expressed HPV58L1 protein; 2, sf301 negtive control; 4, sf301:ΔvirG negtive control.

Invasion ability of recombinant strain HPV58-attenuated *Shigella*

The number of bacteria invaded into HeLa cells was enumerated following the cells were incubated with HPV58-attenuated *Shigella* and the wild-type strain sf301, respectively. The results showed that the intracellular bacterial number in the recombinant strain was much lower compared with the wild-type strain sf301, indicating that the invasion ability of the recombinant strain HPV58-attenuated *Shigella* was greatly diminished but not completely abolished. This is a prerequisite for a candidate vaccine (data not shown).

Production of antibody against HPV58L1

After primary immunization for 13 days, the serum levels of HPV58L1-IgG and IgA were determined. The titers of HPV58L1-IgG and IgA antibody in the PBS group and the sf301 group were all about 1:400−550, and were both ∼1:800 in the immunization group after primary immunization. The immunized animals with HPV58-attenuated *Shigella* showed considerable production of antibody specific to HPV58L1 as the serum level of anti-HPV58L1-IgA and IgG was obviously increased after booster immunization. The titers of anti-HPV58L1-IgA and -IgG reached 1:1540 and 1:1780, respectively. Significant differences in mean serum titer were found between two routes (13 days after immunization and 20 days after immunization) (IgG, $P < 0.05$; IgA, $P < 0.05$). The results demonstrated that the recombinant live vaccine was able to provoke humoral immunoresponse (**Figs. 4** and **5**).

To rule out the role of sf301-LPS in HPV58L1-stimulated immune response, we determined the levels of IgG and IgA. On Day 13 after immunization with sf301-LPS, the levels of IgG and IgA in the sera were remarkably low and the titer was about 1:200, corresponding with those from the control animals. Interestingly, the level of sf301 LPS-IgG was higher than that of IgA in the sera on Day 20 after immunization. No significant difference in mean serum titer was observed between three routes (unimmunization, 13 days after immunization, and 20 days after immunization) (IgG, $P > 0.05$; IgA, $P > 0.05$). These results suggested that the host immune response against sf301 LPS was low and that did not affect the immune response of the host against HPV58L1 (**Fig. 6**).

Table 1 Vaccine protection against virulent challenge

	No. of eyes with severity rating				Protection rate (no. of animals protected/total) (%)
	0	1	2	3	
Immunization group	5	1	0	0	5/6 (83)
Control group	0	0	2	4	0/6 (0)

Severity ratings: 0, no disease or mild irritation; 1, mild keratoconjunctivitis or late development and/or rapid clearing; 2, keratoconjunctivitis, but no purulence; 3, fully developed keratoconjunctivitis, with purulence. Protection was defined as follows: no disease at all or mild irritations (score of 0).

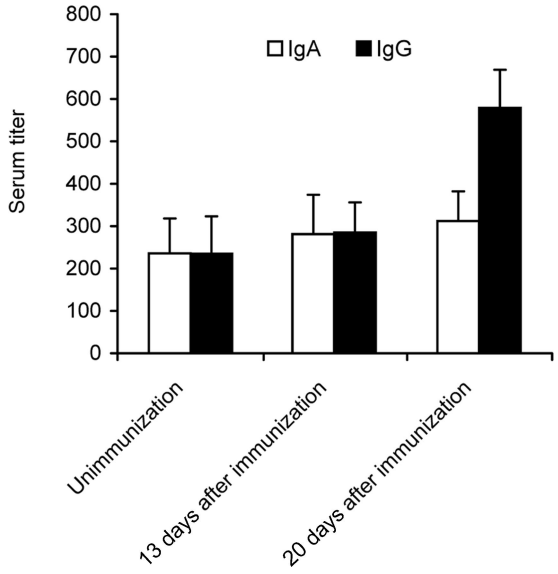

Fig. 4 Titers of serum IgA and IgG antibodies against the immunization group sf301:ΔvirG bacterial antigen (LPS) Six animals from this group were analyzed. Data are expressed as mean ± SD.

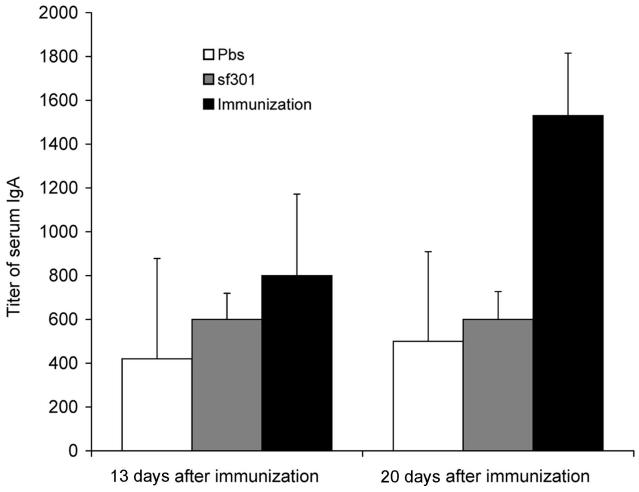

Fig. 5 Titer of serum IgA antibody against HPV58L1 Six animals from each group were analyzed. Data are expressed as mean ± SD.

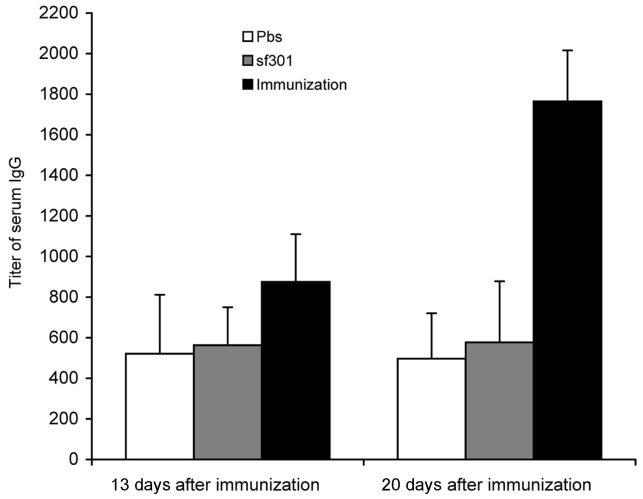

Fig. 6 Titer of serum IgG antibody against HPV58L1 Six animals from each group were analyzed. Data are expressed as mean ± SD.

Measurement of ASC in the spleen and regions lymph nodes of infected animals

HPV58L1-specific IgG- and IgA-ASCs in the spleen, pp, and regional lymph nodes were measured by ELISPOT. In comparison with the sf301 and PBS control groups, animals in the HPV58L1-*Shigella* group displayed significantly increased HPV58L1-specific IgG- and IgA-ASCs in the aforementioned lymphoid tissues. The majority of HPV58L1-specific ASCs were distributed in the MDLN, in which HPV58L1-specific IgA- and IgG-ASC counts reached 420 per 2×10^5 and 500 per 2×10^5 cells, showing that enough mucosal immune was induced. The MSLN, SVCLN, and pp, which were distal to the site of infection, contained only a few specific ASCs (total ASC counts were about $78/2 \times 10^5$ cells). As for the sf301 and PBS groups, only a few of specific ASCs ($45/2 \times 10^5$ cells and $30/2 \times 10^5$ cells, respectively) could be found. There were significant differences in mean ASC counts between the immunization group and the control group (IgG-ASCs, $P < 0.01$; IgA-ASCs, $P < 0.01$) (**Figs. 7** and **8**).

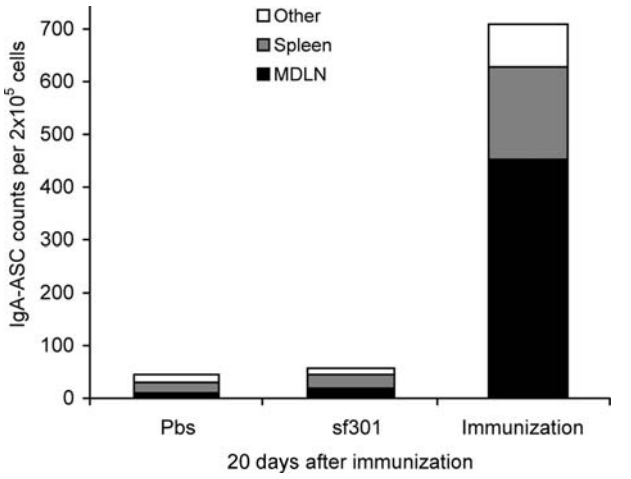

Fig. 7 HPV58L1-specific IgA-ASC counts per 2×10^5 cells 20 days after immunization Three groups (PBS group, sf301 group, and immunization group) were analyzed. Six animals from each group were analyzed. For each animal, positive IgA-ASC counts were determined for the MDLN, the spleen, and other parts (MSLN, SVCLN, and pp). ASC, antibody-secreting cell.

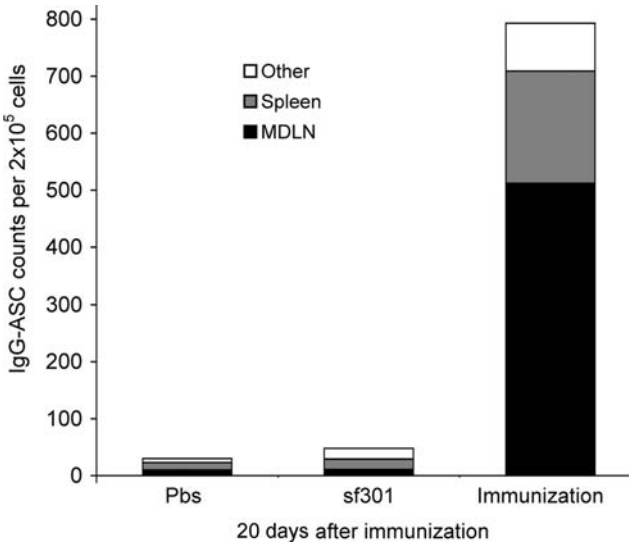

Fig. 8 HPV58L1-specific IgG-ASC counts per 2×10^5 cells 20 days after immunization Three groups (PBS group, sf301 group, and immunization group) were analyzed. Six animals from each group were analyzed. For each animal, positive IgG-ASC counts were determined for the MDLN, the spleen, and other parts (MSLN, SVCLN, and pp). ASC, antibody-secreting cell.

Discussion

As the majority of HPV infections are sexually transmitted diseases, and HR-HPVs infect the human body, especially for women, via mucosa of genital tract, mainly vagina and cervix, therefore, to get the maximal immune protection of female genital tract, it is essential to choose mucosal immunity route for vaccination. Since mucosal immunity is an integral entity, the immune effectors including cells and molecules generated from immunoresponse at the site of any local mucosa can distributed everywhere in the whole mucosal system. The existing HPV vaccine inoculated by subcutaneous or intramuscular injection has been proven effective, but costly. Particularly, it is not convenient. Still, it might not be able to invoke vagina mucosal immunity as strong and optimal as mucosal vaccination. Another point we should emphasize is that HPV58 is the second popular one of HR-HPVs in China and unfortunately there has been no HPV58 prophylactic vaccine studied so far. To produce more advanced HPV vaccine, low-cost, convenient, and easy to use, invoking strongest vagina mucosal immune protection, to produce prophylactic vaccine to HPV58, we chose *Shigella* as a vehicle and inserted HPV58 L1 gene into its genome to develop a live vaccine. In the present study, the results were promising.

Different strategies can be used to deliver vaccine antigen by the mucosal route. Among them, the use of bacterial carriers probably is most potential one. The use of live attenuated bacterial vaccine strains allows the targeted delivery of foreign gene to mammalian cells and tissues via the mucosal route. Delivery of vaccine antigens by live bacterial carriers has resulted in the elicitation of effective humoral and cellular immunoresponse. Attenuated strains of *Shigella* have also shown promise as live vaccine vectors carrying foreign antigen [14,15]. In our previous study, we also fulfilled that HPV type 16L1 capsid protein vaccine was delivered by live attenuated *Shigella flexneri* strain sh42, and the candidate vaccine could stimulate an efficient immune response in mucosal sites of guinea pig [16].

Host's immune response to *Shigella* infection has long been studied. The data suggest that the humoral immune response is a major component of protective immunity to shigellosis with both systemic and mucosal immune responses activated against the LPS and some virulence plasmid-encoded proteins after administration via oral mucosal route. Its low immunogenicity, targeting inductive sites, easy administration, and low delivery-associated costs, undoubtedly, make the *Shigella* as a desirable delivery vehicle [17].

For recombinant live vector vaccine, protection against vehicle pathogen itself and immune response specific for the exologous antigens, i.e. recombinant targeting antigen could be simultaneously achieved. The efficacy of an attenuated live vaccine relies on a subtle

balance between minimal reactogenicity and maximal immunogenicity. The essential question is that the attenuated live bacteria used as a vehicle should not elicit efficient immunoresponse and at the same time should not cover over the immunoresponse to the carried target gene [18,19]. In our experiment, 20 day after immunization, the animals produced anti-HPV58L1-IgG, IgA antibodies conspicuously and intensively, but the antibody to sf301 LPS was very slight and inconsiderable, which is just what we want, indicating that *Shigella* used as a carrier of recombinant HPV58 live vector was ideal.

In the present study, recombinant HPV58L1-attenuated *Shigella* was successfully constructed, HPV58 L1 protein expression was confirmed by western blot, showing a novel extra protein band at 60 kDa. Moreover, the expressed HPV58L1 protein was identified to be able to self-assemble into VLPs by transmission electromicroscopy, and form HPVL1 protein conformation by murine hemagglutination. All of these results indicated that HPV58L1-attenuated *Shigella* live vaccine indeed could generate bioactive HPV58L1 protein.

The ability of Shigellae to invade the corneal epithelia of guinea pigs and to spread to contiguous cells, causing keratoconjunctivitis, provides an ideal model system to test the virulence of *Shigella* strains and the protective efficacy and immunogenicity of *Shigella* vaccines [20]. Animal immunization results showed that there was no keratoconjunctivitis occurred in the immunization group, serum anti-HPV58L1-IgG and -IgA levels were obviously increased, and the lymphoid tissues, especially the local ones, had more HPV58L1-specific ASC. All of these data implied that the candidate vaccine could stimulate an efficient immune response in mucosal site of guinea pig.

Previous study showed that measurement of the local immune response in lymphoid tissue close to the site of immunization might be a better predictor of vaccine efficacy and immunogenicity than measurement in distal sites [20]. In this study, we analyzed the HPV58L1-specific IgG-ASC and IgA-ASC in lymph nodes and spleen, especially in MDLN and spleen of the immunized animals. The results indicated that the lymphocytes specifically generating antibodies against HPV58L1 were predominately distributed in MDLN, the nearest group of lymph nodes to the vaccination sites. This is consistent with the conclusion of Hartman *et al.* [20], and also offers the most simple and convenient method for evaluation of vaccine efficacy.

In summary, our preliminary results indicated that HPV58-attenuated *Shigella* live vector vaccine might be an effective protective HPV58 vaccine, but its feasibility and efficacy remained to be characterized. Additionally, the present results proved once again that *Shigella* could be used as a live vehicle for constructing attenuating live vaccine.

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