

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

Wensheng Li<sup>1,2</sup>, Hongli Liu<sup>3</sup>, Xiaofeng Yang<sup>2</sup>, Jin Zheng<sup>2</sup>, Yili Wang<sup>2</sup>, and Lusheng Si<sup>2\*</sup>

<sup>1</sup>Shaanxi Provincial People's Hospital, The Third Affiliated Hospital, Medical College of Xi'an Jiaotong University, Xi'an 710068, China

<sup>2</sup>Institute for Cancer Research, Key Laboratory of Environment and Genes Related to Diseases of Ministry of Education, Xi'an Jiaotong University, Xi'an 710061, China

<sup>3</sup>The First affiliated Hospital, Medical College of Xi'an Jiaotong University, Xi'an 710061, China

\*Correspondence address. Tel: +86-29-82655191; Fax: +86-29-82655499; E-mail: slusheng@yahoo.com

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 TGTCCTGTGGCGCCTAGTGAG-3' (forward primer) and 5'-GGAGAGCTCTTACTTTCGTCCCAAAGGAAA CTGATCTAGATC-3' (reverse primer) for the amplification of HPV58L1 (C-terminus 33 amino acid deleted) were designed, with *salI/sacI* 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 *salI* and *sacI* 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  $\mu$ l 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  $6 \times 10^8$  bacteria per eye. In the PBS group, 20  $\mu$ l PBS was given. Three weeks later, the animals were challenged with sf301 strain at a dose of  $6 \times 10^8$  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 keratoconjunctivitis 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<sub>2</sub> at 37°C to half-confluence. Then, 25  $\mu$ l

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<sub>2</sub> at 37°C for 30 min. The HeLa cells were then washed six times with DMEM, and treated with DMEM containing 50  $\mu$ g/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  $6 \times 10^8$  HPV58L1-attenuated *Shigella* each animal at Days 0, 2, and 3 for three times, the sf301 group was treated with  $6 \times 10^8$  sf301 strains, and the PBS group was treated with 20  $\mu$ l 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  $\mu$ g/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  $\mu$ g/ml gentamicin, and the cell concentration was adjusted to  $2 \times 10^5$  cells/ml.

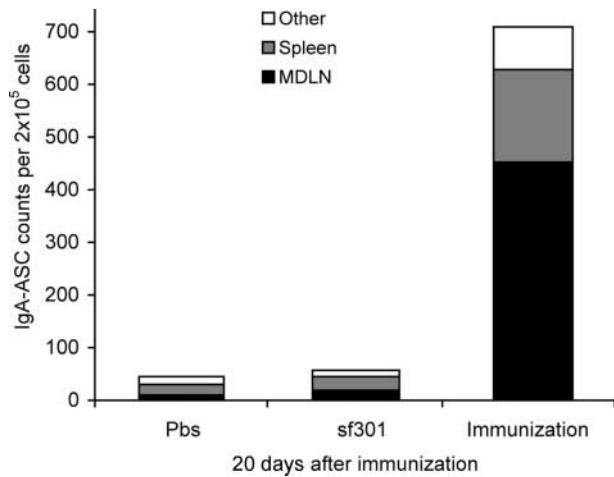
#### **Enzyme-linked immunosorbent spot assay**

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  $\mu$ l HPV58L1 VLP overnight at 4°C. After washing once with PBS, the wells were blocked with 5% fetal

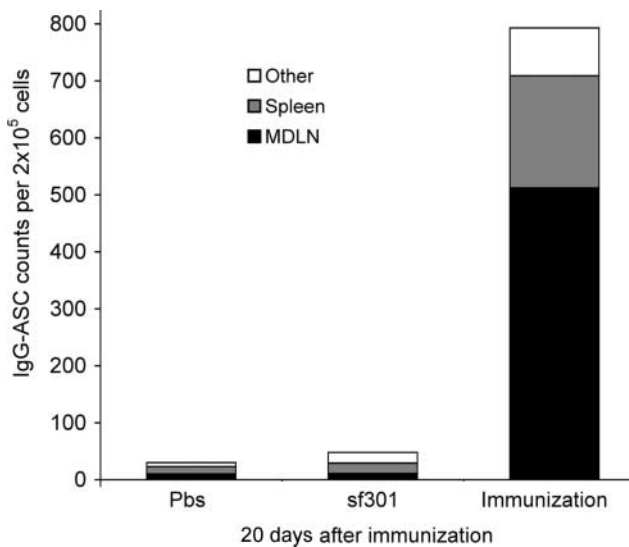








**Fig. 7 HPV58L1-specific IgA-ASC counts per  $2 \times 10^5$  cells 20 days after immunization** Three groups (PBS group, sf301 group, and immunization group) were analyzed. Six animals from each group were analyzed. For each animal, positive IgA-ASC counts were determined for the MDLN, the spleen,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



**Fig. 8 HPV58L1-specific IgG-ASC counts per  $2 \times 10^5$  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 immunore-sponse 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 immunore-sponse. 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 exogenous 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 electron microscopy, and form HPV L1 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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