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Fusion of HPV L1 into *Shigella* surface IcsA: A new approach in developing live attenuated *Shigella*-HPV vaccine

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ABSTRACT

Despite the success of L1 virus-like particles (VLPs) vaccines in prevention of high-risk human papillomavirus (HPV) infection and cervical cancer, extraordinary high cost for the complete vaccination has impeded widespread use of the vaccine in resource-poor countries, where cervical cancers impose greater challenge. Presentation of HPV L1 protein by attenuated pathogenic bacteria through natural infection provides a promising low-cost and convenient alternative. Here, we describe the construction and characterization of attenuated L1-expressing *Shigella* vaccine candidate, by fusion of L1 into the autotransporter of *Shigella sonnei*, IcsA, an essential virulence factor responsible for actin-based motility. The functional α domain of IcsA was replaced by codon-optimized L1 gene with independent open reading frames (ORFs) facilitated by suicide vector pJCB12. The L1 gene was stabilized in the genome of recombinant *S. sonnei* with protein expression and assembly of VLPs in the bacterial cytoplasm. Through conjunctival route vaccination in guinea pigs, L1-containing *S. sonnei* was able to elicit specific immune response to HPV16 L1 VLP as well as bacterial antigens. The results demonstrated the feasibility of the novel stratagem to develop prophylactic *Shigella*-HPV vaccines.

1. Introduction

Infection of high-risk types of human papillomavirus (HPVs) is the major cause of cervical cancer, the second leading malignancy of women worldwide (Bosch et al., 2002; Garland et al., 2007; Snoeck, 2006). Currently, the L1 virus-like particles (VLPs) vaccines have been used in prevention of HPV infection and related neoplastic disease (Campo and Roden, 2010; de Borba et al., 2009; Garland et al., 2007; Kirnbauer et al., 1992; Rose et al., 1993). The two licensed L1 VLP vaccines (Gardasil, Merck & Co., Inc., and Cervarix, GlaxoSmithKline), produced in eukaryotic system, are against two dominant oncogenic HPV genotypes, HPV16 and HPV18, which together account for about 70% of cervical cancers (Campo and Roden, 2010; Harper et al., 2004; Koutsky et al., 2002). However, high cost of these HPV vaccines hinders the application of these vaccines in resource-poor countries which have 80% cervical cancer cases (Campo and Roden, 2010). Thus, cost-effective new vaccines will be the key to widespread vaccination for control of cervical cancer.

The virus capsid L1 protein can be expressed and self-assembled into functional pentamers and even VLPs in bacteria (Baud et al., 2004a; Fraillery et al., 2007; Mustafa et al., 2009; Nardelli-Haefliger et al., 1997; Revaz et al., 2001; Yuan et al., 2001), which offer a cheaper and practical alternative to VLP-based vaccines. Previous studies have demonstrated the feasibility of L1 expression and self-assembly in attenuated pathogenic bacteria like *Salmonella* as well as *Shigella* from plasmid vectors. Immunization of small animals by these recombinant bacteria could stimulate specific immune responses to HPV VLPs (Baud et al., 2004a; Baud et al., 2004b; Fraillery et al., 2007; Yang et al., 2005). However, the instability of the plasmid vectors *in vivo* without antibiotics selection has become obstacle in the further improvement of these vaccine candidates (Nardelli-Haefliger et al., 1997; Revaz et al., 2001). For improving the stability of the plasmid vector and optimized expression of L1, attempts have been made for codon optimization (Baud et al., 2004b) and change of antibiotic marker in *Salmonella* (Fraillery et al., 2007). However, we reasoned that integration of the L1 gene into the bacterial genome can covercome the requriement of antibiotic selection and improve stability of the viral gene in bacterial host.

Shigella causes diarrhea by invading and destroying colorectum epithelium. The bacterium is able to proliferate inside the cell cytosol and to spread intra- and inter-cellularly via the action of IcsA, a surface protein abundantly expressed in all virulent *Shigella* strains (Goldberg et al., 1994). Disruption of IcsA leads to loss of actin-based motility, resulting in markedly reduced virulence in

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humans and animal models (Lett et al., 1989; Schroeder and Hilbi, 2008). In this study, we replaced part of the IcsA coding sequence with the HPV16 L1 gene, which achieved high expression of the viral VLP and attenuation of the bacterial virulence. The resultant vaccine strains were potent in eliciting immune responses to HPV as well as to *Shigella sonnei* via conjunctiva route in guinea pigs.

2. Methods and materials

2.1. Bacterial strains, plasmids, and culture conditions

Bacterial strains and plasmids used in this work are listed in Table 1. *Escherichia coli* and *S. sonnei* strains were routinely cultured on L-agar and L-agar containing Congo red (0.1%), respectively, at 37 °C. Antibiotics (Sigma) were used as follows: streptomycin 100 μg/ml; chloramphenicol 20 μg/ml.

2.2. DNA manipulations

Chromosomal and plasmid DNA was extracted for various *in vitro* manipulations. Oligonucleotides were ordered from Sigma and listed in Table 2. Polymerase chain reaction (PCR) was performed by Gene Engine (Bio-Rad) using Hotstar mixture (Qiagen). Bacterial transformation by electroporation or heatshock was performed as described previously (Calvin and Hanawalt, 1988).

2.3. Construction of icsA-HPV16 L1 transcriptional gene fusion

The coding sequence of residues 105–506 (Charles et al., 2001) of *S. sonnei* IcsA (http://www.ncbi.nlm.nih.gov/nuccore/NC_007385.1) was replaced with entire HPV16 L1 gene (http://www.ncbi.nlm.nih.gov/nuccore/JX897004.1). To construct the gene fusion, 3 fragments were PCR amplified: the first fragment included 312 bp of the *icsA* 5'-end coding sequence and 550 bp upstream non-coding sequence with a stop codon at the 5'-end by primers P1F and P1R; the second fragment was the entire coding sequence of HPV16 L1 gene by primers P2F and P2R with independent SD sequence 11 bp in front of the L1 start codon at the 5'-end and a stop codon at the 3'-end; the third fragment included the coding sequence of *icsA*, bp 1519–3306, by primers P3F and P3R. All the fragments were sequentially ligated and cloned into pUC19 using In-Fusion™ Advantage PCR Cloning Kit (Clontech) to generate pUC19-*icsA*-L1. In the resultant construct, the transcription of L1 gene was driven by the *icsA* promoter and its translation was initiated via the independent SD sequence. Since the L1 gene was not in-frame with either side of the *icsA* coding sequence and therefore it could produce intact L1 protein.

Table 2
Oligonucleotides used in this study.

Name	Sequence	Target
P1F	CGGTACCCGGGGATCTAGTTATGTTTGATGTCTGCA	*icsA*
P1R	CTG TTTCCTGTAATTTAAAGT TCTAGATGCATGAGAGG	*icsA* (outframe)
P2F	AATTACAGGAAACAGGTATGAGCCTGTGGCTGCCCA	HPV16 L1 (outframe)
P2R	ACTTCATTTAACAGCTTCCTCTTCTTCCTC	HPV16 L1 (outframe)
P3F	GCTGTTAAATGAAGTACTATTCTGGCAGATAAT	*icsA* (outframe)
P3R	CGACTCTAGAGGATCTCAGAAGGTATATTTCACAC	*icsA*
SP1F	GGTGCATCCCTGTTGCTCG	*icsA*
SP1R	TGGGCACCAGGATCTTGTTG	HPV16 L1
SP2F	ACACCTTCTGGGAGGTGAAC	HPV16 L1
SP2R	CCAGCGGTACGTGTCATAGC	*icsA*
P19F	GATCCTCTAGAGTCGACCTGC	Linearized pUC19
P19R	GATCCCCGGGTACCGAGCT	Linearized pUC19

The gene fusion construct (Fig. 1A) was subcloned into the suicide vector pJCB12, to give rise to pJCB12-*icsA*-L1. The subclone was maintained in *E. coli* SM10λ*pir*, which supports the replication of R6K origin plasmids and allows the expression of the plasmid-borne *tra* genes for conjugation.

2.4. Construction of the Shigella-HPV vaccine strain

This was achieved via conjugation. A streptomycin resistant derivative SS86St^r of a wildtype *S. sonnei* strain SS86 was isolated as recipient strain and the *E. coli* SM10λ*pir*/pJCB12-*icsA*-L1 as the donor. As illustrated in Fig. 1B, a single crossover was achieved by selecting chloramphenicol and a double crossover was achieved by sucrose selection as described by Turner (Turner et al. (2006), Turner et al. (2001)). The resultant colonies were analysed by PCR-electrophoresis (Fig. 1C as well as DNA-sequencing of the PCR products to confirm the second crossover and the presence of the constructed gene fusions.

2.5. Cell infection and fluorescent imaging

Gentamycin-killing assay was performed as previously described (Lucchini et al., 2005). Briefly, HEp-2 (ATCC CCL-23) cells were cultured in Dulbecco's minimal essential medium (DMEM) (Sigma–Aldrich) containing 10% fetal bovine serum with 5% CO_2 at 37 °C and seeded in 24-well plates and cultured to ~80% confluence. *S. sonnei* strains from the mid-exponential phase were added to the cells at an MOI (*m*ultiplicity *o*f *i*nfection) of 10 (for CFU

Table 1
Bacterial strains and plasmids.

Name	Relevant genotype, phenotype, and description	Source/reference[a]
Strains		
DH5α		Promega
SM10λ*pir*	*pir tra*RP4	SI
SS86	*S. sonnei* (wild-type *S. sonnei*)	SRL
SS86St^r	Strepmycin resistant derivative of SS86	This work
SS86St^r/L1	*S. sonnei* with HPV16 L1 infused with *icsA* in transcriptional-fusion ORFs	This work
SS86Δ*icsA*/p322L1	*S. sonnei* with deletion in *icsA* harboring plasmid carrying L1 viral gene	
Plasmids		
pUC19	2690 bp plasmid for infusion PCR fragments, *ori*UC, *cat*, *lacZ*, Linearized vector generated by PCR	Clontech
pUC19-*icsA*-L1	4168-bp fragment, including *icsA* gene with HPV16 L1 in transcriptional-fusion ORFs, in pUC19	This work
pJCB12	oriR6 K mobRP4 sacBcat	SI
pJCB12-*icsA*-L1	4168-bp fragment, including *icsA* gene with HPV16 L1 in transcriptional-fusion ORFs, in pJCB12	This work

[a] SI, provided by Dr. Arthur K. Turner from The Wellcome Trust Sanger Institute, Cambridge, UK; SRL, provided by Scottish *Salmonella* and *Shigella* Reference Laboratory, Glasgow, UK.

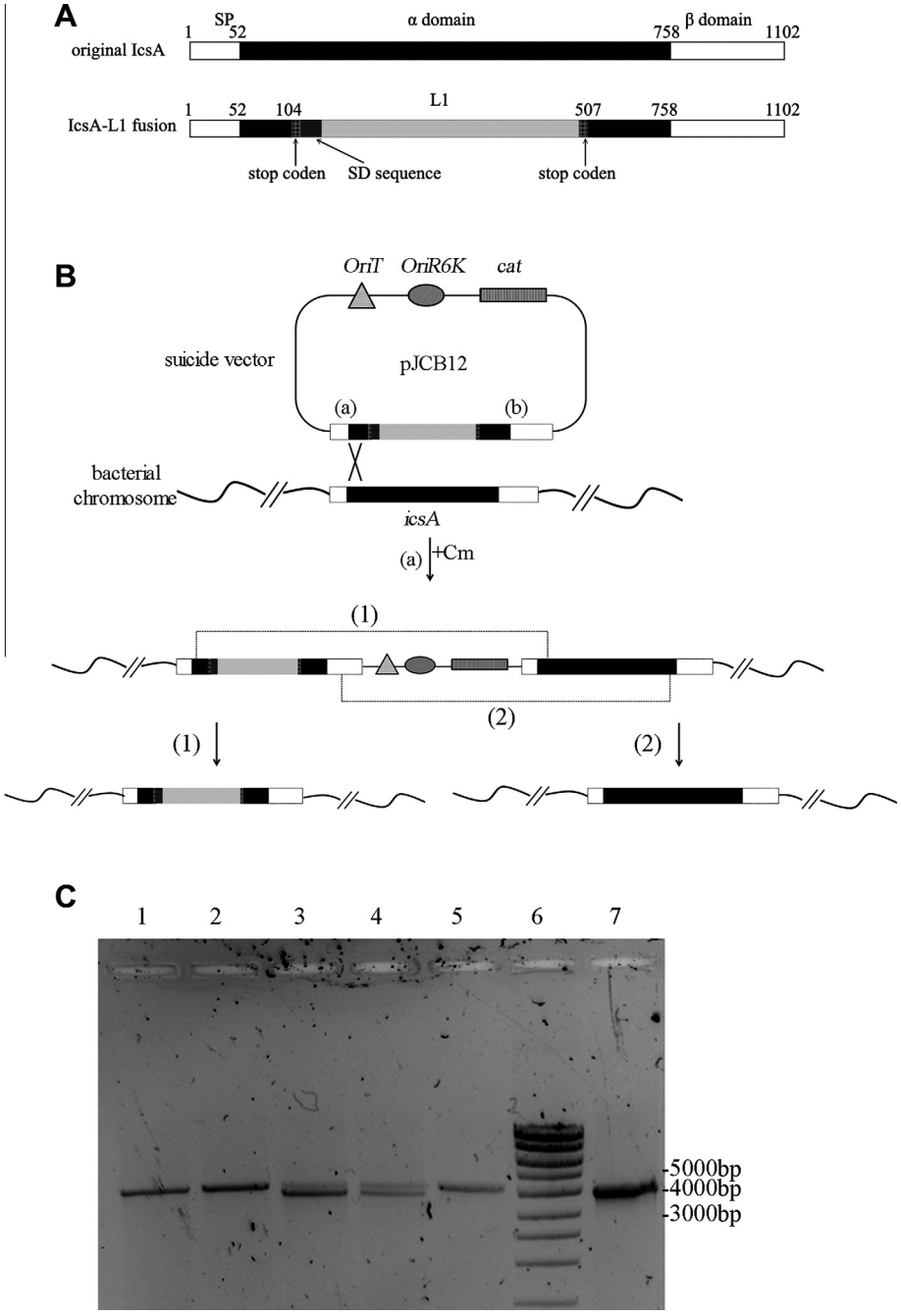

Fig. 1. Construction of the vaccine strain SS86St^r/L1. (A) Schematic illustration of the original and recombinant IcsA structure. Note that L1 coding sequence is not in-frame with the *icsA* coding sequences and stop codens were present at the 5'- and 3'-ends of the L1 gene. There is SD sequence 11 bp in front of the start conden of the L1 gene. (B) The *icsA*-L1 fusion was subcloned to give rise to pJCB12-*icsA*-L1, which was used to replace the wildype *icsA* gene in the large virulence plasmid of *S. sonneivia* by conjugation and homologous recombination. The single crossover was achieved by selecting chloramphenicol resistance and double crossover was achieved by selecting sucrose resistance as described (Turner et al., 2001). Note that double crossovers could resolve to wildtype *icsA* (1) or *icsA*-L1 fusion in the genome (2). The *icsA*-fusion is slightly larger in size than the wildtype *icsA*, which can be distinguished by PCR and DNA electrophoresis shown in (C): Lane 1 and 2, product from vaccine strains; lane 3 and 4, products after single crossover. Note that there are two bands corresponding to the *icsA*-L1 fusion (upper band) and wildtype *icsA* (lower band) respectively; lane 5, product from pJCB12-*icsA*-L1; lane 6, DNA molecular marker; lane7, product from the streptomycin-resistant recipient *S. sonnei* strain.

counting) or 100 (for immunofluorescence imaging). *Shigella* bacteria were centrifuged at 2000 r.p.m. for 10 min at room temperature to the cells. After 40 min incubation, extracellular bacteria were removed and the cells were washed with PBS. DMEM containing gentamicin (50 μg ml^{-1}) was added for further incubation. At appropriate time intervals, cells were lysed for CFU counting or fixed for fluorescence imaging. For CFU counting, the infected cells monolayer was washed with PBS and lysed with 0.1% Triton X-100 in water. The cell lysates were plated onto LB agar to determine the number of CFU. For fluorescence imaging, cells were fixed with 3.7% paraformaldehyde for 10 min at room temperature. After permeabilized with 0.1% Triton-100 (in PBS) for 3 min and blocked with 5% BSA (in PBS) for 30 min at room temperature, cells were treated with anti-HPV16 L1 monoclonal antibody [289–16981] (Abcam) overnight at 4 °C followed by goat anti-mouse antibody conjugated with TRITC (Invitrogen) for 1 h at room temperature.

Coverslips were mounted with Anti-Fade solution (Invitrogen) and images were analyzed with a Leica Confocal microscope (Germany).

2.6. Transmission Electron Microscopy (TEM)

The recombinant *S. sonnei* strain was grown to late log phase ($OD_{600} \geqslant 0.8$) in 200 ml L-broth and the bacteria were harvested by centrifuge at 5000*g* for 20 min. The pellet was suspended in 5 ml of ice-cold PBS containing 0.5 M NaCl and a protease inhibitor cocktail (Complete). The bacteria was disrupted by sonication with six 20-s pulses and centrifuged at 8000*g* for 20 min at 4 °C. Supernatant were collected and applied slowly to the top of a 10 to 40% sucrose gradient (8 ml) prepared in PBS–0.5 M NaCl buffer. The gradient was centrifuged at 224,000*g* in a SW41Ti rotor (Beckman) at 4 °C for1.5 h and then fractionated into 23 aliquots from the bottom to the top of the centrifuge tube. Heavier L1-positive fractions (30–40% sucrose) were stained with phosphotungstic acid and examined by TEM (H-600, HITACHI).

2.7. Production of HPV L1 VLPs

HPV16 or 58 L1 VLPs were produced in insect cells harboring HPV L1-containing recombinant baculovirus as described by Zheng et al. (2004). Briefly, HPV16 or 58 L1 gene was cloned into a pFastBac donor plasmid, and the recombinant plasmid was transformed into DH10Bac competent cells, which contain the bacmid, with a mini-attTn7 target site and the helper plasmid. The mini-Tn7 element on the pFastBac donor plasmid can transpose to the mini-attTn7 target site on the presence of transposition proteins provided by the helper plasmid. Colonies containing recombinant bacmids were identified by disruption of the lacZ gene. High molecular weight mini-prep DNA was prepared from selected *E. coli* clones containing the recombinant bacmid, and this DNA was then used to transinfect insect Sf9 cells which were maintained in supplemented Grace's insect medium with 10% fetal calf serum to produce recombinant baculoviruses. Sf9 cells were infected with these recombinant baculoviruses at a multiplicity of infection (MOI) of 20. Cells were harvested 72 h postinfection and fractionated by sonication with three 15-s bursts at 60% maximal power (Vibra-cell) in ice-bath with the presence of protease inhibitor cocktails (Complete). His-tag, from pFastBac plasmid, was used to purify VLPs using Probond™ column (Invitrogen) under native condition following the manufacturer's instructions.

2.8. Immunization of guinea pigs and analysis of immune responses

Guinea pigs were immunized with the vaccine *S. sonnei* strain via conjunctival route as described (Labrec et al., 1964). The protocol has been proved by the Animal Research Ethical Committee of the Xi'an Jiaotong University. Briefly, female guinea pigs aged 6–8 weeks old, weighing 200–300 g, were divided into 5 groups with 4 animals in each group. Animals of the conjunctival group were given *Shigella*-HPV vaccine with 5×10^8 CFU per eye of bacteria from mid-log phase culture and were observed for 5–6 days for development of the conjunctivitis (Philpott et al., 2000). Animals of the subcutaneous group were injected subcutaneously with 10-μg HPV16 VLPs in PBS. Immunization was boosted with the same dosages two weeks after. The immunization procedures would be expected to cause no more than minimal and transient pain in a fully conscious animal. Guinea pigs were sacrificed six weeks after the second immunization. Blood and vaginal secretions were collected for antibodies detection, and lymphocytes from spleen were obtained for ELISPOT assay as previously described (Yang et al., 2005).

Enzyme linked immunosorbent assay (ELISA) and enzyme linked immunospot (ELISPOT) assay was performed to evaluate HPV16 VLP-specific antibodies and ASC (*a*ntibody-secreting *c*ells) frequency in the spleens, respectively (Nardelli-Haefliger et al., 1997; Yang et al., 2005). Neutralization assays (NA) were performed with secreted alkaline phosphatase (SEAP) HPV16 pseudoviruses as described by Buck et al. (2005), Fraillery et al. (2007). Briefly, purified SEAP HPV16 pseudoviruses diluted 1600-fold were incubated on ice for 1 h with two-fold serial dilutions of the guinea pig serum, and the pseudovirus-antibody mixtures were used to infect 293TT cells, which was then cultured at 37 °C with 5% CO_2 for 60 h. The SEAP content in 10 μl of clarified cell supernatant was determined using the Report Assay Kit – SEAP-(TOYOBO). Neutralization titers were defined as the reciprocal of the highest serum dilution that caused at least a 50% reduction in SEAP activity. The lowest serum dilutions tested were 1:100. At this dilution, guinea pig serum of the negative control group did not show significant neutralizing activity.

3. Results

3.1. Construction and verification of the icsA-L1 gene fusion in recombinant S. sonnei

The overall strategy for construction of recombinant *S. sonnei* is outlined in Fig. 1A and B. The *icsA*-L1 fusion was constructed by a sequential fusion procedure as described in materials and methods. The wildtype *icsA* was replaced with the *icsA*-L1 fusion via conjugation and homologous recombination as detailed in the figure legends. The presence of the *icsA*-L1 gene fusion in recombinant *S. sonnei* was confirmed by PCR using *icsA*-specific primers, P1F and P3R, and DNA electrophoresis. Fig. 1C shows clearly that the PCR product of the *icsA*-L1 fusion is slightly larger in size than that of the wildtype *icsA*, which are 4168 and 3809 bp, respectively. PCR-sequencing has further confirmed the presence of designed upstream and downstream junctions between HPV16 L1 and *icsA* genes (data not shown). One recombinant strain SS86St^r/L1 (Table 1) was chosen as the vaccine candidate for further evaluation in the following experiments.

3.2. The stability and expression of L1 gene in strain SS86St^r/L1

icsA-L1 gene fusion was inserted into pBR322 (p322L1) and electroporated into an *icsA*-deleted *S. sonnei* strain to generate SS86Δ*icsA*/p322L1 (Table 1). Stability of p332L1 was compared with the *icsA*-L1 fusion that was integrated in the genome, by continuous sub-culturing. Overnight cultures were sub-cultured at 1/100 dilution in L-broth with or without ampicillin. The overnight cultures were also plated out on L-agar in the presence or absence of ampicillin to estimate the loss of p332L1. In the absence of ampicillin, most of bacteria lost p322L1 in three days (Fig. 2A). This data suggested that p332L1 would not be stably maintained during ocular inoculation where antibiotic pressure did not exist. To test this postulation, SS86Δ*icsA*/p322L1 was inoculated to guinea pig eyes and recovered three days later from the eyes. Colony PCR was carried out to detect L1 gene as the evidence of p332L1 existence. The result showed that only ~3% of bacteria retained the p332L1 plasmid. In contrast, the same PCR procedure revealed that ~90% of the vaccine strain, SS86Str/L1, retained L1 gene (Fig. 2B).

The expression of L1 protein under the native promoter of *icsA* in strain SS86St^r/L1 was initially confirmed by immunoblotting using HPV16 L1-specific monoclonal antibody (Fig. 2C). The expression of the L1 protein was further confirmed by fluorescence imaging. The L1 protein, as indicated by the red fluorescence signals, was found to be evenly distributed in the bacterial cells when

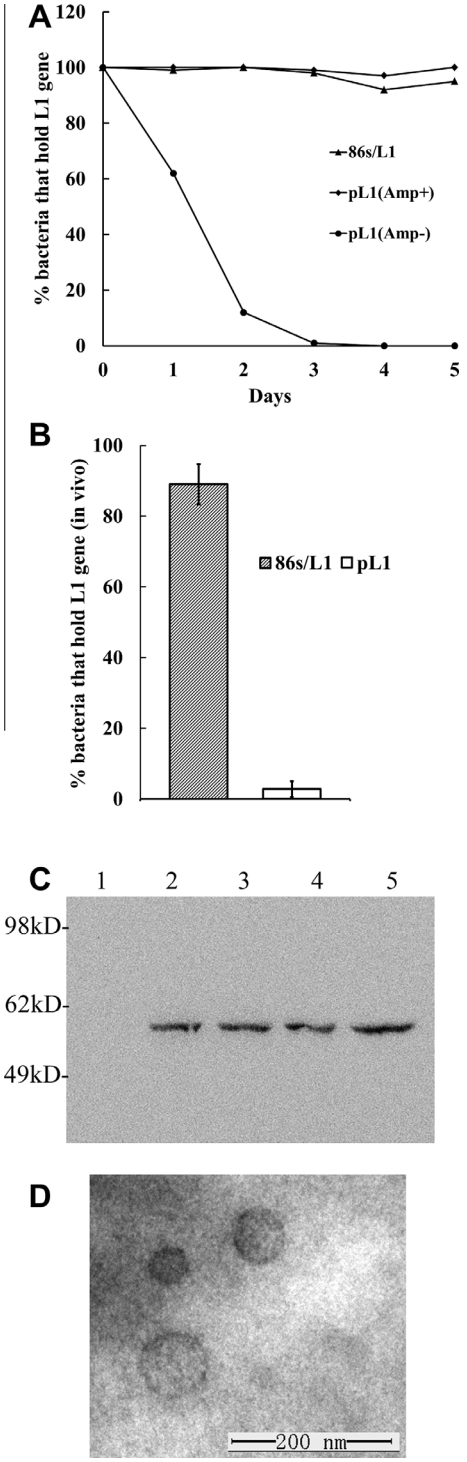

Fig. 2. Stability and expression of the L1 gene in strain SS86St^r/L1. *In vitro* (A) and *in vivo* (B) stability of the L1 gene in genome-integrated vaccine strain SS86St^r/L1 (86s/L1) (without antibiotics) and plasmid-bearing *S. sonnei* strain SS86Δ*icsA*/p322L1 (pL1) was determined by colony PCR. For *in vitro*, the number of successive cultures at a 1/100 dilution performed overnight in medium with (Amp+) or without (Amp−) antibiotic is indicated on the horizontal axis. Each morning, bacteria were plated onto agar in the presence or absence of antibiotic. For *in vivo*, bacteria were recollected from the eyes of immunized animals three days after the initial inoculation. The percentage of the bacteria that retained L1 gene as indicated by the vertical axis. (C) Western blotting for the expression of L1 in the vaccine strain. Lane 1, total protein from the wildtype strain 86; Lane 2–5, total protein collected from strain SS86St^r/L1 after passage at day 0, day 1, day 5 and day 10, respectively. (D) TEM images of the assembled VLPs in strain SS86St^r/L1. The sizes of produced VLPs were heterogeneous, ranging from 30–60 nm. Bar = 200 nm.

cultured in broth as well as when the bacteria were inside host cells (Fig. 3). To determine whether the L1 protein was assembled into VLPs strain SS86St^r/L1, the bacterial lysate was fractionated through a 10–40% sucrose gradient and the heavier fractions, containing the L1 protein, were analyzed by TEM. Capsomers with typical spherical particles were revealed from the bacterial lysate, which appeared heterogeneous in size, with diameters ranging from 30 to 60 nm (Fig. 2D).

3.3. The fitness and virulence properties of strain SS86St^r/L1

Growth curve of all the strains was plotted by the optical density at 600 nm every 30 min until late stationary phase. Compared with the wildtype parental strain, SS86Δ*icsA*/p322L1 requried 2 h more to reach optical density of 2.0, while the vaccine strain, SS86Str/L1, grew eaqually fast as the wildtype strains (Fig. 4A). The gentamycin-killing assay was used to evaluate invasiveness of strains SS86St^r/L1 with an MOI of 10. Two hours after invasion, cells were lysed to recover intracellular CFU. The results showed that strain SS86St^r/L1 remained invasive despite a statistically insignificant drop ($p > 0.05$) of the intracellular CFU in comparison with the wild-type parental strain at the time interval (Fig. 4B).

In Sereny test, wild-type *S. sonnei* strain was able to cause severe conjunctivitis to guinea pigs at the dose of 5×10^5 organisms in 5 days (Fig. 4C top). Strain SS86St^r/L1 could barely cause irritation although 1000-fold more (5×10^8) organisms were used in conjunctival inoculation (Fig. 4C bottom).

3.4. Immune responses elicited by strain SS86St^r/L1 via conjunctival immunization in guinea pigs

Strain SS86St^r/L1 (5×10^8 CFU) was administrated through the conjunctival route at day 1 and day 14. Sera, vaginal secretions, and spleen lymphocytes were collected 8 weeks after the primary conjunctival inoculation. The levels and specificity of the immune responses to the viral L1 protein and the bacterial LPS was determined by specific ASCs. The results showed that, 8 weeks after immunization, SS86St^r/L1 elicited more than 100-fold increase of ASC frequency specific to HPV16 L1 VLP in the spleen lymphocytes; the wildtype parental strain, 86, produced background noise of ASC frequency comparable to that of the PBS negative controls. On the other hand, both strain 86 and strain SS86St^r/L1 elicited significant and comparable levels of LPS-specific ASC frequencies compared to the PBS-induced background noise (Fig. 5A).

3.5. Detection of antibody specific to VLPs

Sera were collected from guinea pigs 8 weeks after immunisation with strain SS86St^r/L1. ELISA was used to determine the binding properties of the serum IgG to baculovirus-derived VLPs in PBS (native VLPs) or in carbonate buffer (pH 9.5) (disassembled VLPs). The IgG elicited by strain SS86St^r/L1 strongly recognized native VLPs but poorly recognized the denatured VLPs as judged by the high and low titers across of serum samples (Table 3). HPV58 VLPs, produced in baculovirus-insect cell system, were also used in VLP-based ELISA. Animal derived anti-sera showed low level of cross-reaction (<10%) was against HPV58 VLPs.

To further evaluate the above vaccination scheme (i.e. SS86St^r/L1 via conjunctival route), we compared it with purified HPV16 VLPs via subcutaneous route. Groups of four guinea pigs were immunized with two doses of SS86St^r/L1 (5×10^8 CFU) at days 0 and 14 (Fig. 5B, squares) through conjunctival route or two subcatneous doses of 10-μg VLPs at day 0 and 14 (Fig. 5B, trian-

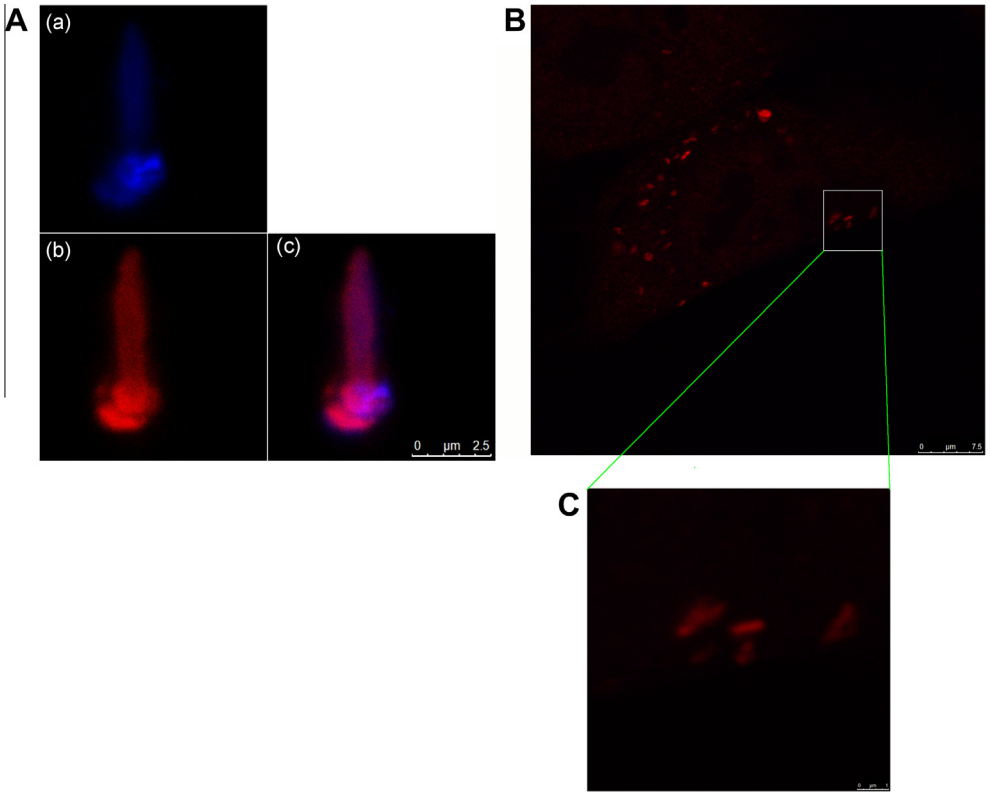

Fig. 3. Fluorescent microscopy on strain SS86St^r/L1 in L-broth (A) and inside HEp-2 cells (B and C). (A) Bacterial genome DNA is stained with DAPI (blue), the L1 protein is stained in red by anti-L1 and goat anti-mouse conjugated with TRITC. (B and C) Cofocal image of strain SS86St^r/L1 inside HEp-2 cells. The same antibodies are used as in A; all intracellular bacteria are stained in red. C is enlarged window in B. Bars depict the amplification. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

gles). Our data showed both vaccination schemes elicited HPV16-neutralizing antibodies at comparable levels (Fig. 5B).

4. Discussion

In the development of prophylactic HPV vaccine based on attenuated bacterial vector, stably maintaining the viral protein-expression in the vaccine strains has been a key issue for immunization. When plasmids are used for viral protein expression, frequent loss of the plasmids has been observed, especially *in vivo*, despite various options have been exploited to improve the stability of the plasmids (Fraillery et al., 2007). In light of this problem, we have sought to integrate the HPV16 L1 gene to the bacterial genome. We choose *S. sonnei* as the host and its *icsA* locus as the integration site for several considerations. Firstly, similar to *Salmonella* spp., *Shigella* also represents an ideal vehicle to deliver HPV L1 protein to mucosal sites. As important diarrhea-causing pathogens, *Shigella* can invade the colonrectum epithelium of humans and primates. *Shigella* bacteria can express and carry the viral capsid protein cross the lumen of the gut through M cells in Peyer's patches and then be taken up by macrophages and dendritic cells at local sites through the natural infection. An innate immune response, triggered by the short and self-limited *Shigella* infection, could facilitate the development of adaptive immune responses specific to the virus. These responses triggered by mucosal delivery can be effective at both mucosal and systemic sites. Furthermore, *Shigella* infection is restricted in the colonrectum epithelium and does not cause carrier state. In contrast, other organisms, such as *Salmonella* spp., may disseminate into other tissues and cause carrier state, which are safety concerns in terms of vaccination. Secondly, *icsA* gene is located on the virulence plasmid of *S. sonnei*, which is a stable component of the bacterial genome. Integration and expression of viral gene in this locus would be more stable compared with expression of viral using a manmade plasmid. As a major virulence determinant, IcsA is expressed abundantly in *Shigella*; replacement of IcsA coding sequence with viral genes should attenuate *Shigella* virulence and result in stably expression of the viral protein under the *icsA* promoter (Lett et al., 1989).

Importantly, compared with plasmid-based system, integration of the L1 gene in the genome did not cause growth defect, and the viral gene was maintained and expressed over a 10 day consecutive passage *in vitro*, Moreover, most of the bacteria recovered from the eyes of inoculated animals maintained the viral gene in their genome (Fig. 2A and B). These results have demonstrated the advantages of integration of the viral gene in the genome over expressing the viral gene using plasmid vectors.

The L1 DNA sequence used in our study is the codon-optimized version described by Baud et al. in 2004, which increased the stability of the L1-expressing plasmid and improved immunogenicity in *Salmonella* (Baud et al., 2004b). We assumed that the *Salmonella*-optimized HPV16 L1 would also be optimal for expression in *Shigella* due to the close genetic relationship between *Salmonella* and *Shigella*. We engineered a transcriptional fusion with SD sequence 11-bp in front of the L1 gene start codon, which resulted in stably expression of the viral protein as demonstrated by immunoblotting and fluorescent microscopy (Figs. 2 and 3). Furthermore, the viral protein was assembled into capsomers structure as shown by TEM (Fig. 2D). Previous studies have showed that L1 pentamers expressed in *E. coli* and *Salmonella* could display conformational epitopes that elicit neutralizing antibodies (Yuan et al., 2001). Consistent with these reports, we observed that

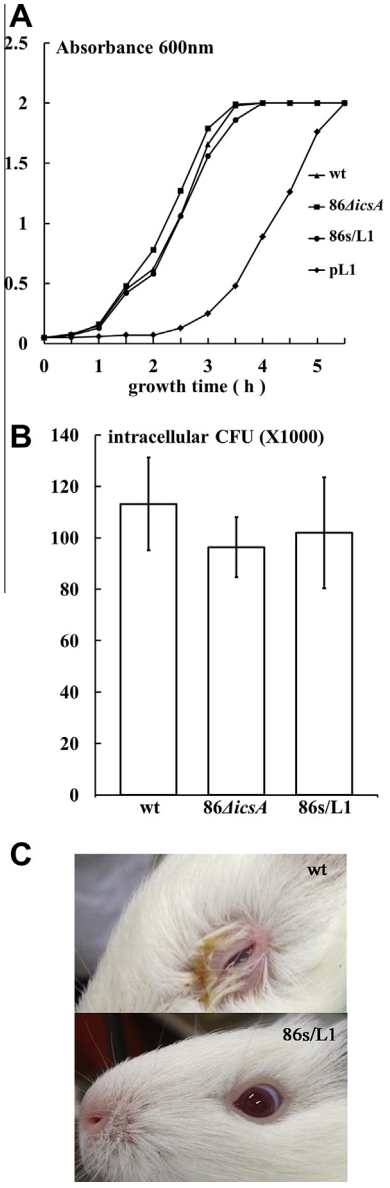

Fig. 4. Fitness of the vaccine strain SS86St^r/L1. (A) Growth curve of the wild-type *S. sonnei* strain (wt), *icsA*-deleted *S. sonnei* strain (86Δ*icsA*), genome-integrated vaccine strain SS86St^r/L1 (86s/L1) and plasmid-bearing *S. sonnei* strain SS86Δ*icsA*/p322L1 (pL1). Y axis indicates the optical density of the cultures (600 nm). X axis indicates time (h). (B) Intracellular CFU were recovered from cell lysates 2 h after cell invasion. Three independent experiments were carried out and one representative set of data is presented. All samples were in triplicate and error bars are shown. (C) Sereny test. Wildtype *S. sonnei* causes grade-3 keratoconjunctivits in 5 days (top). The vaccine strain SS86St^r/L1 induced minimal irritation (bottom).

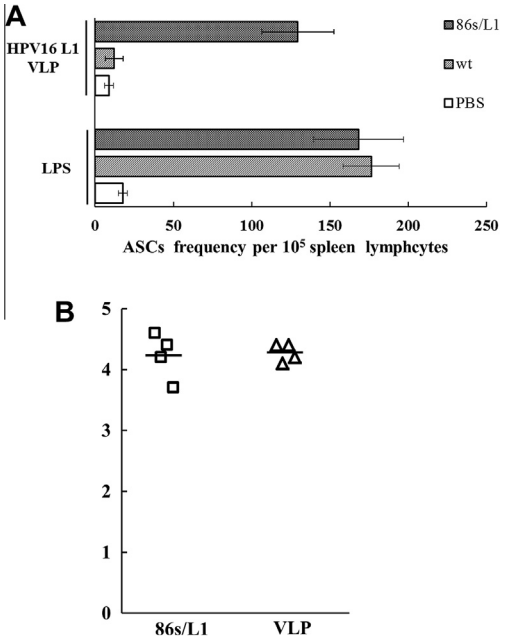

Fig. 5. Immunological analysis of the vaccinated guinea pigs. HPV16 L1 VLP-specific IgG ASCs and LPS-specific IgG ASCs were determined from 10^5 spleen lymphcytes (A). PBS was used as negative controls. All samples were in triplicate and error bars are shown. HPV16-neutralizing antibody titers from animals immunized witht two doses of SS86St^r/L1 (5×10^8 CFU) at days 0 and 14 (squares) through conjunctival route and two s.c. doses of 10-μg VLPsat day 0 and 14 (triangles) were determined by SEAP HPV16 pseudovirion neutralization assay (B). Data are expressed as the $\log_{10}$ of the reciprocal dilutions yielding 50% SEAP inhibition for the neutralizing titers. Between-group differences were analyzed with a Student's t test.

Table 3
Serum IgG ELISA titers of the guinea pigs immunized by strain SS86St^r/L1.

Guinea pig	Native HPV16 VLP	Denatured HPV16 VLP	Native HPV58 VLP
#1	25600	200	320
#2	12800	100	1280
#3	6400	<100	640
#4	12800	100	320

anti-sera exhibited high titers to native VLPs but lower titers to dissembled VLPs in ELISA, which indicated that the majority of *Shigella*-derived L1 proteins were folded into ordered structures of epitopes suitable for eliciting neutralizing antibodies. The potential of SS86St^r/L1 as a *Shigella* vaccine was seemingly not impaired by the expression of HPV16 VLPs, demonstrated by significant proliferation of HPV16 VLP-specific and *S. sonnei* LPS-specific ASCs as well in the spleens.

Type-specific response to HPV L1 VLP vaccine has been considered as an important aspect to evaluate the vaccine candidates. The type-restricted prophylactic immunity to a range of papillomaviruses was firstly reported by Jarrett et al. (1990). And then serological evidence confirmed that the immunodominant neutralizing antibodies generated by L1 VLPs are type-specific and are not cross-neutralizing (Rose et al., 1994; Rose et al., 1998; White et al., 1998). Immunological studies with VLPs have shown that antibodies cross-reactive with multiple HPV VLP types recognize type-common epitopes and are, in general, linear and non-neutralizing (Fleury et al., 2006; Orozco et al., 2005). Highly homologous VLPs such as HPV-6/11, HPV-31/33, HPV-18/45, and HPV-16/31 share one or more cross-neutralization epitopes (Giroglou et al., 2001). However, these cross-neutralization epitopes appear to be less immunogenic than the type-specific epitopes (Combita et al., 2002). We used HPV58 VLPs (produced in insect cell-baculovirus system) as controls in VLP-based ELISA to investigate their cross-reaction with the antiserum from the immunized guinea pigs. As shown in Table 3, low level of cross-reaction (<10%) was detected against HPV58 VLPs. Our observations were largely consistent with the previously reports mentioned above.

HPV L1 VLP vaccines provides necessary positive control to evaluate new HPV vaccines due to their verified efficacy. Experiments, preclinical and clinical trials have provided solid evidence that VLPs could stimulate high-titer neutralizing antibodies that can prevent further HPV infection of the same type (Campo and Roden, 2010; de Borba et al., 2009; Harper et al., 2004; Kirnbauer et al., 1992; Schiller et al., 2008). We have demonstrated again the effectiveness of VLP subcutaneous vaccination, and SS86St^r/

L1 via ocular route was equally effective (Fig. 5B). However, currently licensed vaccines have been optimized for maximal efficacy, which are more superior than the VLPs used in this study (Garçon et al., 2011; Revaz et al., 2007). From this perspective, *Shigella*-HPV vaccine requires improvement for immunogenicity.

While invasiveness is required for optimal deliver the viral antigen (see above arguments) the live bacteria must be sufficiently attenuated so that the bacteria will not cause disease upon vaccination. Disruption of IcsA has been considered to be an essential step in engineering live attenuated *Shigella* vaccines (Goldberg et al., 1994; Kotloff et al., 2000; Lett et al., 1989). more importantly, disruption of IcsA would not undermine the ability of *Shigella* to invade host cells. Previous studies have revealed that the entry of *Shigella* into epithelial cells (invasion) is mediated by the type III secretion apparatus and Ipa proteins of *Shigella*, and IcsA does not play a role in this process (Schroeder and Hilbi, 2008; Tran Van Nhieu and Sansonetti, 1999; Yu, 1998). The replacement of *icsA* coding sequence with the L1 viral gene appeared to have tipped the balance between bacterial virulence attenuation and viral protein expression and immunogenicity in our model systems. The vaccine strain remained invasive in gentamicin-killing assay (Fig. 4B), which was presumably important for eliciting observed immune responses (Fig. 5, Table 3). On the other hand, the vaccine strain was adequately attenuated in the guinea pig eye keratoconjunctivitis model (Fig. 4C).

In summary, our study demonstrated that the feasibility of using live attenuated *S. sonnei* as carrier to deliver HPV16 L1 antigen for vaccination against *Shigella* as well as HPV infections. This approach offers an attractive alternative novel strategy for developing cost-effective vaccines against HPV infections for prevention of cervical cancers. Although IcsA-based attenuation is sufficient for safely use in the keratoconjunctivitis model, further genetic modification will be required to improve safety when testing in primate models and human subjects according to previous studies (Hartman and Venkatesan, 1998; Kotloff et al., 2000). Investigations are under the way to further improve the described vaccine strain and test the new constructs in guinea pig eye as well as other animal models.

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References

Baud, D., Benyacoub, J., Revaz, V., Kok, M., Ponci, F., Bobst, M., Curtiss III, R., De Grandi, P., Nardelli-Haefliger, D., 2004a. Immunogenicity against human papillomavirus type 16 virus-like particles is strongly enhanced by the PhoPc phenotype in *Salmonella enterica serovar Typhimurium*. Infect. Immun. 72, 750–756.

Baud, D., Ponci, F., Bobst, M., De Grandi, P., Nardelli-Haefliger, D., 2004b. Improved efficiency of a *Salmonella*-based vaccine against human papillomavirus type 16 virus-like particles achieved by using a codon-optimized version of L1. J. Virol. 78, 12901–12909.

Bosch, F., Lorincz, A., Munoz, N., Meijer, C., Shah, K., 2002. The causal relation between human papillomavirus and cervical cancer. J. Clin. Pathol. 55, 244–265.

Buck, C.B., Pastrana, D.V., Lowy, D.R., Schiller, J.T., 2005. Generation of HPV pseudovirions using transfection and their use in neutralization assays. Methods Mol. Med. 119, 445.

Calvin, N., Hanawalt, P., 1988. High-efficiency transformation of bacterial cells by electroporation. J. Bacteriol. 170, 2796–2801.

Campo, M.S., Roden, R.B.S., 2010. Papillomavirus prophylactic vaccines: established successes, new approaches. J. Virol. 84, 1214–1220.

Charles, M., Pérez, M., Kobil, J.H., Goldberg, M.B., 2001. Polar targeting of Shigella virulence factor IcsA in Enterobacteriacae and Vibrio. Proc. Nat. Acad. Sci. 98, 9871.

Combita, A.L., Touzé, A., Bousarghin, L., Christensen, N.D., Coursaget, P., 2002. Identification of two cross-neutralizing linear epitopes within the L1 major capsid protein of human papillomaviruses. J. Virol. 76, 6480–6486.

de Borba, P., Naud, P., Roteli-Martins, C., De Carvalho, N., Teixeira, J., Aoki, F., Ramjattan, B., Romanowski, B., Shier, R., Somani, R., 2009. Sustained efficacy and immunogenicity of the human papillomavirus (HPV)-16/18 AS04-adjuvanted vaccine: analysis of a randomised placebo-controlled trial up to 6.4 years. Lancet 374, 1975–1985.

Fleury, M., Touze, A., Alvarez, E., Carpentier, G., Clavel, C., Vautherot, J.F., Coursaget, P., 2006. Identification of type-specific and cross-reactive neutralizing conformational epitopes on the major capsid protein of human papillomavirus type 31. Arch. Virol. 151, 1511–1523.

Fraillery, D., Baud, D., Pang, S.Y.Y., Schiller, J., Bobst, M., Zosso, N., Ponci, F., Nardelli-Haefliger, D., 2007. *Salmonella enterica serovar Typhi* Ty21a expressing human papillomavirus type 16 L1 as a potential live vaccine against cervical cancer and typhoid fever. Clin. Vaccine Immunol. 14, 1285–1295.

Garçon, N., Morel, S., Didierlaurent, A., Descamps, D., Wettendorff, M., Van Mechelen, M., 2011. Development of an AS04-adjuvanted HPV vaccine with the adjuvant system approach. BioDrugs 25, 217–226.

Garland, S., Hernandex-Avila, M., Wheeler, C., 2007. Cervical cancer and HPV vaccination. N. Engl. J. Med. 356, 1915–1927.

Giroglou, T., Sapp, M., Lane, C., Fligge, C., Christensen, N.D., Streeck, R.E., Rose, R.C., 2001. Immunological analyses of human papillomavirus capsids. Vaccine 19, 1783–1793.

Goldberg, M.B., Theriot, J.A., Sansonetti, P.J., 1994. Regulation of surface presentation of IcsA, a *Shigella* protein essential to intracellular movement and spread, is growth phase dependent. Infect. Immun. 62, 5664.

Harper, D.M., Franco, E.L., Wheeler, C., Ferris, D.G., Jenkins, D., Schuind, A., Zahaf, T., Innis, B., Naud, P., De Carvalho, N.S., 2004. Efficacy of a bivalent L1 virus-like particle vaccine in prevention of infection with human papillomavirus types 16 and 18 in young women: a randomised controlled trial. Lancet 364, 1757–1765.

Hartman, A.B., Venkatesan, M.M., 1998. Construction of a stable attenuated *Shigella sonnei* ΔvirG vaccine strain, WRSS1, and protective efficacy and immunogenicity in the guinea pig keratoconjunctivitis model. Infect. Immun. 66, 4572–4576.

Jarrett, W., O'Neil, B., Gaukroger, J., Laird, H., Smith, K., Campo, M., 1990. Studies on vaccination against papillomaviruses: a comparison of purified virus, tumour extract, and transformed cells in prophylactic vaccination. Vet. Rec. 126, 449–452.

Kirnbauer, R., Booy, F., Cheng, N., Lowy, D., Schiller, J., 1992. Papillomavirus L1 major capsid protein self-assembles into virus-like particles that are highly immunogenic. Proc. Nat. Acad. Sci. 89, 12180.

Kotloff, K.L., Noriega, F.R., Samandari, T., Sztein, M.B., Losonsky, G.A., Nataro, J.P., Picking, W.D., Barry, E.M., Levine, M.M., 2000. *Shigella flexneri* 2a strain CVD 1207, with specific deletions in virG, sen, set, and guaBA, is highly attenuated in humans. Infect. Immun. 68, 1034–1039.

Koutsky, L.A., Ault, K.A., Wheeler, C.M., Brown, D.R., Barr, E., Alvarez, F.B., Chiacchierini, L.M., Jansen, K.U., 2002. A controlled trial of a human papillomavirus type 16 vaccine. N. Engl. J. Med. 347, 1645–1651.

Labrec, E.H., Schneider, H., Magnani, T.J., Formal, S.B., 1964. Epithelial cell penetration as an essential step in the pathogenesis of bacillary dysentery. J. Bacteriol. 88, 1503–1518.

Lett, M., Sasakawa, C., Okada, N., Sakai, T., Makino, S., Yamada, M., Komatsu, K., Yoshikawa, M., 1989. VirG, a plasmid-coded virulence gene of *Shigella flexneri*: identification of the virG protein and determination of the complete coding sequence. J. Bacteriol. 171, 353.

Lucchini, S., Liu, H., Jin, Q., Hinton, J.C.D., Yu, J., 2005. Transcriptional adaptation of *Shigella flexneri* during infection of macrophages and epithelial cells: insights into the strategies of a cytosolic bacterial pathogen. Infect. Immun. 73, 88–102.

Mustafa, W., Maciag, P.C., Pan, Z., Weaver, J.R., Xiao, Y., Isaacs, S.N., Paterson, Y., 2009. Listeria monocytogenes delivery of HPV-16 major capsid protein L1 induces systemic and mucosal cell-mediated $CD4^+$ and $CD8^+$ T-cell responses after oral immunization. Viral Immunol. 22, 195–204.

Nardelli-Haefliger, D., Roden, R., Benyacoub, J., Sahli, R., Kraehenbuhl, J.P., Schiller, J.T., Lachat, P., Potts, A., De Grandi, P., 1997. Human papillomavirus type 16 virus-like particles expressed in attenuated *Salmonella typhimurium* elicit mucosal and systemic neutralizing antibodies in mice. Infect. Immun. 65, 3328–3336.

Orozco, J.J., Carter, J.J., Koutsky, L.A., Galloway, D.A., 2005. Humoral immune response recognizes a complex set of epitopes on human papillomavirus type 6 L1 capsomers. J. Virol. 79, 9503–9514.

Philpott, D.J., Edgeworth, J.D., Sansonetti, P.J., 2000. The pathogenesis of *Shigella flexneri* infection: lessons from *in vitro* and *in vivo* studies. Philos. Trans. R. Soc. Lond. B Biol. Sci. 355, 575–586.

Revaz, V., Benyacoub, J., Kast, W.M., Schiller, J.T., De Grandi, P., Nardelli-Haefliger, D., 2001. Mucosal vaccination with a recombinant *Salmonella typhimurium* expressing human papillomavirus type 16 (HPV16) L1 virus-like particles (VLPs) or HPV16 VLPs purified from insect cells inhibits the growth of HPV16-expressing tumor cells in mice. Virology 279, 354–360.

Revaz, V., Zurbriggen, R., Moser, C., Schiller, J.T., Ponci, F., Bobst, M., Nardelli-Haefliger, D., 2007. Humoral and cellular immune responses to airway immunization of mice with human papillomavirus type 16 virus-like particles and mucosal adjuvants. Antiviral Res. 76, 75–85.

Rose, R., Bonnez, W., Reichman, R., Garcea, R., 1993. Expression of human papillomavirus type 11 L1 protein in insect cells: *in vivo* and *in vitro* assembly of virus like particles. J. Virol. 67, 1936–1944.

Rose, R.C., Bonnez, W., Da Rin, C., McCance, D.J., Reichman, R.C., 1994. Serological differentiation of human papillomavirus types 11, 16, and 18 using recombinant virus-like particles. J. Gen. Virol. 75, 2445–2449.

Rose, R.C., White, W.I., Li, M., Suzich, J.A.A., Lane, C., Garcea, R.L., 1998. Human papillomavirus type 11 recombinant L1 capsomeres induce virus-neutralizing antibodies. J. Virol. 72, 6151–6154.

Schiller, J.T., Castellsagué, X., Villa, L.L., Hildesheim, A., 2008. An update of prophylactic human papillomavirus L1 virus-like particle vaccine clinical trial results. Vaccine 26, K53–K61.

Schroeder, G.N., Hilbi, H., 2008. Molecular pathogenesis of *Shigella* spp.: controlling host cell signaling, invasion, and death by type III secretion. Clin. Microbiol. Rev. 21, 134.

Snoeck, R., 2006. Papillomavirus and treatment. Antiviral Res. 71, 181–191.

Tran Van Nhieu, G., Sansonetti, P.J., 1999. Mechanism of *Shigella* entry into epithelial cells. Curr. Opin. Microbiol. 2, 51–55.

Turner, A.K., Beavis, J.C., Stephens, J.C., Greenwood, J., Gewert, C., Thomas, N., Deary, A., Casula, G., Daley, A., Kelly, P., 2006. Construction and phase I clinical evaluation of the safety and immunogenicity of a candidate enterotoxigenic *Escherichia coli* vaccine strain expressing colonization factor antigen CFA/I. Infect. Immun. 74, 1062–1071.

Turner, A.K., Terry, T.D., Sack, D.A., Londoño-Arcila, P., Darsley, M.J., 2001. Construction and characterization of genetically defined aro omp mutants of enterotoxigenic *Escherichia coli* and preliminary studies of safety and immunogenicity in humans. Infect. Immun. 69, 4969–4979.

White, W.I., Wilson, S.D., Bonnez, W., Rose, R.C., Koenig, S., Suzich, J.A., 1998. *In vitro* infection and type-restricted antibody-mediated neutralization of authentic human papillomavirus type 16. J. Virol. 72, 959–964.

Yang, X.F., Qu, X.Z., Wang, K., Zheng, J., Si, L.S., Dong, X.P., Wang, Y.L., 2005. Construction of prophylactic human papillomavirus type 16 L1 capsid protein vaccine delivered by live attenuated *Shigella flexneri* strain sh42. Acta Biochim. Biophys. Sin. 37, 743–750.

Yu, J., 1998. Inactivation of DsbA, but not DsbC and DsbD, affects the Intracellular survival and virulence of *Shigella flexneri*. Infect. Immun. 66, 3909–3917.

Yuan, H., Estes, P.A., Chen, Y., Newsome, J., Olcese, V.A., Garcea, R.L., Schlegel, R., 2001. Immunization with a pentameric L1 fusion protein protects against papillomavirus infection. J. Virol. 75, 7848–7853.

Zheng, J., Ma, J., Yang, X.-F., Liu, H.-L., Cheng, H.-W., Si, L.-S., Wang, Y.-L., 2004. Highly efficient and economical baculovirus expression system for preparing human papillomavirus type16 virus-like particle. Acta Biochim. Biophys. Sin. 36, 548–552.