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Construction, identification, and immunogenic assessments of an HSV-1 mutant vaccine with a *UL18* deletion

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Summary. – Herpes simplex virus type 1 (HSV-1) is a mucosal and nerve pathogen, whose morbidity shows an increasing tendency. Although several antiviral drugs exist, there is no cure for viral latency for virtually all carriers. There is an urgent need for an HSV-1 vaccine to control infection and limit its spread and recurrence. The *UL18* gene, encoding a vital component of capsids, is one of the essential genes of HSV-1. Deletion of *UL18* from HSV-1 may be exploited as a new approach to develop an attenuated vaccine. The purpose of this study was to construct a DNA vaccine with a full-length *UL18* gene deletion of the HSV-1 genome that can induce an effective immune response. A *UL18*-knockdown plasmid (BAC-HSV-1ΔUL18) was constructed using the bacterial markerless gene knockout system, consisting of the functional pREDI plasmid and BAC-HSV-1 plasmid. Mice were immunized weekly for 3 weeks, and at 1 week post immunization, blood and splenocyte samples of vaccinated and control groups of mice were prepared for immunogenicity assessment. The level of immune response was evaluated using a DTH assay, cytokine determination, and splenocyte proliferation assay. Combination of the pREDI plasmid and BAC-HSV-1 plasmid provides an effective bacterial markerless gene knockout system. Using two-step homologous recombination with the UL18 homologous recombination fragment constructed by multistep PCR amplification, BAC-HSV-1ΔUL18 plasmid vaccine was successfully constructed and was found to significantly enhance cellular immune responses.

Keywords: homologous recombination; UL18 gene; HSV-1; gene knockout; immunogenicity

Introduction

As an extremely common pathogen worldwide, HSV-1 causes infections primarily of the lip, pharynx, eye, central nervous system, and, occasionally, genitals. Moreover, HSV-1 has a certain relationship with Alzheimer's disease (Piacentini *et al.*, 2014), sporadic encephalitis (Kennedy and Chaudhuri, 2002), AIDS-related complications (Weller, 2011), multiple tumors (Jensen *et al.*, 2010) and disseminated disease (Boivin *et al.*, 2006). HSV is persistent as it establishes a life-long latent infection within the nervous system and eliminates the host immune response. Thus, developing an efficient HSV vaccine is an ideal way to control its infection and limit its spread and recurrence.

HSV-1 is a spherical virus consisting of an envelope, tegument, capsid, and genomic DNA (Grunewald and Cyrklaff *et al.*, 2006). Among the eleven glycoproteins located on the HSV-1 envelope, gD and gB have been shown to be important HSV-1 immunogens, inducing cellular and humoral immunity

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Abbreviations: BAC = bacterial artificial chromosome; BAC-HSV-1 = the HSV-1 strain 17 genome inserted into the UL37/UL38 inter genic region; BAC-HSV-1ΔUL18 = full-length *UL18* gene deletion of the HSV-1 genome plasmid; DTH = delayed type hypersensitivity; HSV-1 = herpes simplex virus type 1

(Jin *et al.*, 2001). The capsid, one of the most crucial structural components for protecting DNA, is an icosahedron composed of 11 pentamer molecules and 150 hexamer molecules of capsid protein VP5. VP23, encoded by the *UL18* gene, is an essential capsid protein, and a key component of the triplex structure that links the pentamers and hexamers. The triplex, which contains two units of VP23 and a single unit of VP19C, is important for stabilizing the capsid shell structure (Trus *et al.*, 1996). Interference of protein VP23 greatly affects virus packaging and replication (Jin *et al.*, 2014). In short, HSV-1 with mutant UL18 has the potential to be used to create a DNA vaccine to stimulate immune responses *in vivo*.

DNA vaccines are developed using molecular biology techniques, which make use of the cell system of a host to express an antigen gene from a naked DNA plasmid. As the third generation of vaccines after subunit and attenuated live virus vaccines, DNA vaccines offer immense advantages compared with previous vaccines. DNA vaccines complete antigen synthesis and presentation *in vivo*, which induces immune responses of $CD8^+$ T cells through MHC-I recognition (Rodrigue *et al.*, 1997). The process, by which DNA vaccines invoke immunogenicity, is similar to that of natural viral infection as opposed to that of subunit and attenuated live virus vaccines.

Here, we present the bacterial markerless gene knockout system to produce a *UL18* knockout HSV-1 plasmid (BAC-HSV-1ΔUL18) by using two-step homologous recombination. We further found that BAC-HSV-1ΔUL18 induced a great cellular immune effect in mice. This suggests the potential significance of utilizing the *UL18* knockout HSV-1 DNA vaccine, which can be combined with an attenuated live virus vaccine by using the markerless gene knockout system *in vitro*.

Materials and Methods

Cells, bacteria, and plasmids. African green monkey kidney cells (Vero) (ATCC CCLS1), purchased from American Type Culture Collection, were maintained in Dulbecco's Modified Eagle Medium (Gibco, USA) supplemented with 10% fetal bovine serum (FBS) at 5% CO_2 in a humidified 37°C incubator. Vero cells were used in virus rescue and antibody neutralization assays. *Escherichia coli* K-12 strain MG1655 was used in all markerless gene knockdown experiments. *E. coli* stain DH5α, as a cloning host, was used for plasmid transformation and extraction. All strains were grown in Luria Broth (LB). Plasmids pKD46, BAC, and BAC-HSV-1 are stored in our laboratory. Plasmid pKD46, with Cm^r and Amp^r resistance, carries an arabinose-inducible promoter driving λ-Red recombinase expression. Plasmid BAC-HSV-1, acquired from Gierasch *et al.* (2006) has the BAC sequence inserted into the U_L37/U_L38 intergenic region of HSV-1 strain 17 genome. The bacterial artificial chromosome (BAC) sequence is flanked by cre/LoxP sites with Cm^r resistance, with loxP site-flanked BAC sequences removed by cre recombinase *in vitro*. Then the mixture of BAC and HSV-1 sequences was separated by DNA electrophoresis. Plasmids pREDI (Amp^r) and BAC-HSV-1ΔUL18 (Cm^r) were constructed in our laboratory. Plasmid pREDI was constructed from plasmid pKD46 by insertion of I-SecI-encoded gene under the control of rhamnose promoter PrhaB using the *Nco*I restriction site. The construction strategy of plasmid BAC-HSV-1ΔUL18 is illustrated in the results. The plasmid inducible conditions used were LB with a final concentration of 10 mM arabinose or rhamnose. The lethal concentration of sucrose for the SacB induction site on pREDI is 5% of the LB.

PCR amplification. PCR was performed in a 50 μl reaction mixture containing 25 μl of 2× PrimeSTAR® GC buffer, 4 μl of 2.5 mmol/l dNTP mixture, 1.5 μl each of 20 μmol/l forward and reverse primers, <200 ng of template DNA, 0.5 μl of PrimeSTAR® HS DNA Polymerase (2.5 U/μl, Takara), and up to 50 μl of triple distilled water in a tube (Eppendorf). Reaction conditions consisted of an initial denaturation step at 94°C for 3 min, followed by 30 cycles of 98°C for 1 s, 60°C for 5 s and 72°C for 1 min/kb, with a final extension at 72°C for 5 min. The PCR products were analyzed by 1% agarose gel electrophoresis. The E.Z.N.A gel extraction Kit (Omega Bio-Tek) was utilized for gel extraction. The PCR primer sequences used are as follows: I-SceI: 5'-TTA GAC TGG TCG TAA TGA AAT TCA GCA GGA TCA CAT CTG GGT C-3' and 5'-CAT GCC ATG GGT CGA CTT ATT TCA GGA AAG TTT CGG AGG AGA TAG TG-3'; A-C: 5'-CCC ACC CCC CCG TGG GTC TAG CCG GGC CGG CGC CGA TCCACG CGG CAG G-3'; Kan^r: 5'-TGG CTT TGT TGA ATA AAT CGC GAC CAC GGC CAGAGC GACCCG TCC-3' and 5'-GGA CGG GTC GCT CTG GCC GTG GTC GCG ATT TAT TCA ACA AAG CCA CG-3'; SacB: 5'-CTG GCA ATT ACC CTG TTA TCC CTA GGC CCG TAG TCT GCAAAT CCT TTT-3' and 5'-CAT CGC GAT ACC CTC GGG CAT CTC GCA TCT TGC AAG AATGGG CCT CGT T-3'; A-C-Kan^r-SacB-B: 5'-TGG ATG CCC ACC CCC ACC CCC CCG TGG GTC TAG CCG GGC-3' and 5'-ATG CTG GCG GAC GGC TTT GAA ACT GAC ATC GCG ATA CCC TCG GGC ATC TCG-3'.

Electroporation and purification of plasmids. Electrocompetent recipient cells were prepared from *E. coli* strain DH5α with BAC-HSV-1 and pREDI plasmids. The preparation method was that the *E. coli* strain DH5α with BAC-HSV-1 and pREDI plasmids were washed and resuspended in 10% of the glycerinum solution three times in low temperature conditions. Electroporation shock conditions were 1500 V for 40 μs (Eppendorf). Plasmids were purified using PureLink® HiPure Plasmid Filter DNA Purification Kits (Invitrogen, USA), and stored in sterilized triple-distilled water. The total yields of BAC-HSV-1ΔUL18, BAC and BAC-HSV-1 plasmids were 4.2 mg, 4.2 mg and 1.8 mg, respectively. Meanwhile, the A260/A280 values were greater than 1.8.

Transfection. Vero cells were seeded into 6-well cell culture plates and grown to 70% confluence for plasmid transfection. Plasmids were dissolved in Opti-MEM, siRNA-Mate was subsequently added, and the transfection complex was added directly to Vero cells. Cells were observed for the cytopathic effect.

Immunofluorescence. After transfection, Vero cells were stained with VP5 antibody (Abcam) and DAPI (Beyotime Biotechnology).

Fluorescence images were acquired using an OLYMPUS fluorescence microscope (OLYMPUS IX71).

Mouse immunity. Bilateral musculus biceps brachii injections were conducted in male Kunming mice (Laboratory Animals Monitoring Institute, Guangdong) at 5-weeks of age, with 100 μg per mouse administered once a week for 3 weeks. There were 4 groups, namely the negative control, positive control, experimental group, and normal control, which received BAC, BAC-HSV-1, BAC-HSV-1ΔUL18, and PBS, respectively. One week post the third triplicate-vaccination, blood was collected from the orbits and splenic lymphocytes were extracted in each group.

Sample preparation. One week post final immunization, mice were euthanized by cervical dislocation. To prepare blood samples, eyeballs were extirpated to draw blood, and the blood collected by heparin sodium-anticoagulation tube was centrifuged at 4°C, 2,500 x g for 5 min. The supernatants were extracted for IgG determination and antibody neutralization assays. To collect splenocyte samples, spleens were excised, cut into pieces in DMEM, and digested by adding pancreatin for 30 min. Subsequently, the splenocytes were collected using an 80-mm mesh, centrifuged at 150 x g for 5 min and allowed to rest for 5 min. After addition of NaCl (3.6%; 1 ml), the splenocytes were washed with DMEM twice. These splenocytes were used for the detection of cytokines and in the cell proliferation assays.

Delayed type hypersensitivity assay. One week after the last immunization, the right subcutaneous auricle of vaccinated mice was injected with inactivated HSV-1 suspension (10 μl), and the left was injected with supernatant of the frozen lysate (10 μl). The thickness of auricles was measured 48 h later using a vernier caliper. Data are represented as DTH, which is calculated by subtracting the measurement for the left subcutaneous auricle from the right one.

Determination of cytokines by ELISA. Splenocytes were diluted to a final concentration of 5×10^6 cells/ml in DMEM supplemented with 10% FCS in 24-well plates. Appropriately diluted plasmid solutions were added to the wells (10 μg/well), as well as normal and negative controls. After 48 h, the supernatants of the wells were collected and the levels of cytokines (IL-2, IL-4, L-10, and IFN-γ) in the supernatants of splenocytes from the vaccinated and control groups were measured using the Cytokine ELISA kit, as per manufacturer's instructions.

Determination of IgG by ELISA. IgG was determined in immune mice sera by ELISA method. Briefly, the wells of the flat bottom plates were coated with the inactivated HSV-1 liquid (100 μl /well). 3% of BSA (Fraction V) were added to the wells for 2 h at room temperature, so that the wells are covered. After washing, the plate was probed with appropriately vaccinated sera and pre-immune sera as negative control. This was followed by 1 h incubation with 1:1000 dilution of HRP mutton anti-mouse-IgG conjugate at room temperature, several washes and 100 μl of O-phenylenediamine was added. The reaction was stopped after 10 min by adding 0.2 mol/l of H_2SO_4 and the OD was read at 492 nm.

Cell proliferation assay. Proliferation of splenocytes from the immunized and control groups of mice was measured by the MTT cell proliferation assay. Briefly, the splenocytes were diluted to a final concentration of 5×10^6 cells/ml in DMEM supplemented with 10% FCS in 96-well plates. After 24 h, cells were treated with plasmids (10 μg/ml). After 72 h, the proliferation of the splenocytes was measured by adding MTT. Data are represented as stimulation index (SI), which is calculated by dividing the absorbance (OD) observed in experimental group by the OD observed in negative cells.

Neutralization antibody assay. The mixtures of HSV-1 virus (100 $TCID_{50}$) and BAC-ΔUL18-, BAC-HSV-1- or BAC-vaccinated sera, respectively, by double dilution were added to DMEM supplemented with 2% inactivated FCS. The Vero cells were inoculated in 96-well plates (100 μl /well, 3 repeated wells of one concentration). The plates were subsequently incubated at 37°C for 5 days. Then the degree of cytopathic effect was recorded by observing the Vero cells in an optical microscope (LEICA). The symbol "+" and "-" represents the degree of cytopathic effect. "+", 1%~25% of Vero cells in one well have cytopathic effect; "++", 25%~50% of Vero cells in one well have cytopathic effect; "+++", 50%~75% of Vero cells in one well have cytopathic effect; "++++", 75%~100% of Vero cells in one well have cytopathic effect; "-", none of Vero cells in one well has cytopathic effect.

Statistical analysis. Statistical analysis was performed using Prism 5 software (GraphPad). Two-tailed Student's t-test was used to determine the significance of difference between two groups. *P <0.05; **P <0.01. The data were presented as mean±standard error of the mean.

Results

Construction of the bacterial markerless gene knockout system

The pREDI plasmid is the core of the bacterial gene knockout system, which contains two independent in-

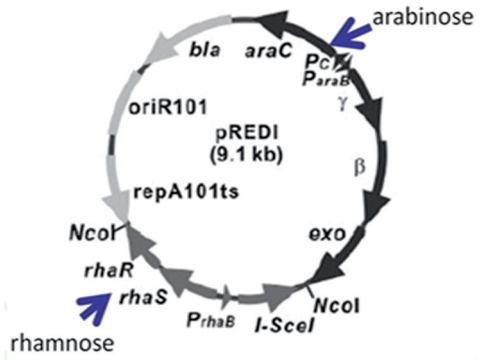

Fig. 1

pREDI plasmid profile

The pREDI plasmid, 9.1 kb in length, provides (i) arabinose-inducible (promoter = ParaB) λ-Red recombinase functions in the replacement of a target genomic region with a linear DNA cassette and (ii) rhamnose-inducible (promoter = PrhaB) I-SceI endonuclease functions in markerless deletion.

ducible promoters to express two different enzymes (Yu *et al.*, 2008): (i) λ-Red recombination protein expression driven by an arabinose-inducible promoter, and (ii) I-SceI endonuclease expression driven by a rhamnose-inducible promoter (Fig. 1), designed for two-step homologous recombination. As the functional plasmids, pREDI plasmids were transformed to *E. coli* with BAC-HSV-1 plasmids, constructing the bacterial markerless gene knockout system.

Construction of UL18-deletion BAC-HSV-1 vaccine

Through the above constructed *in vitro* viral gene knockout system, we deleted the VP23-coding gene by using two-step homologous recombination. Taking the target *UL18* gene as a central point marked by the B gene, the sequence upstream of it was A and that downstream was C. To build the UL18 homologous recombination fragment with forward and reverse screening maker genes, we

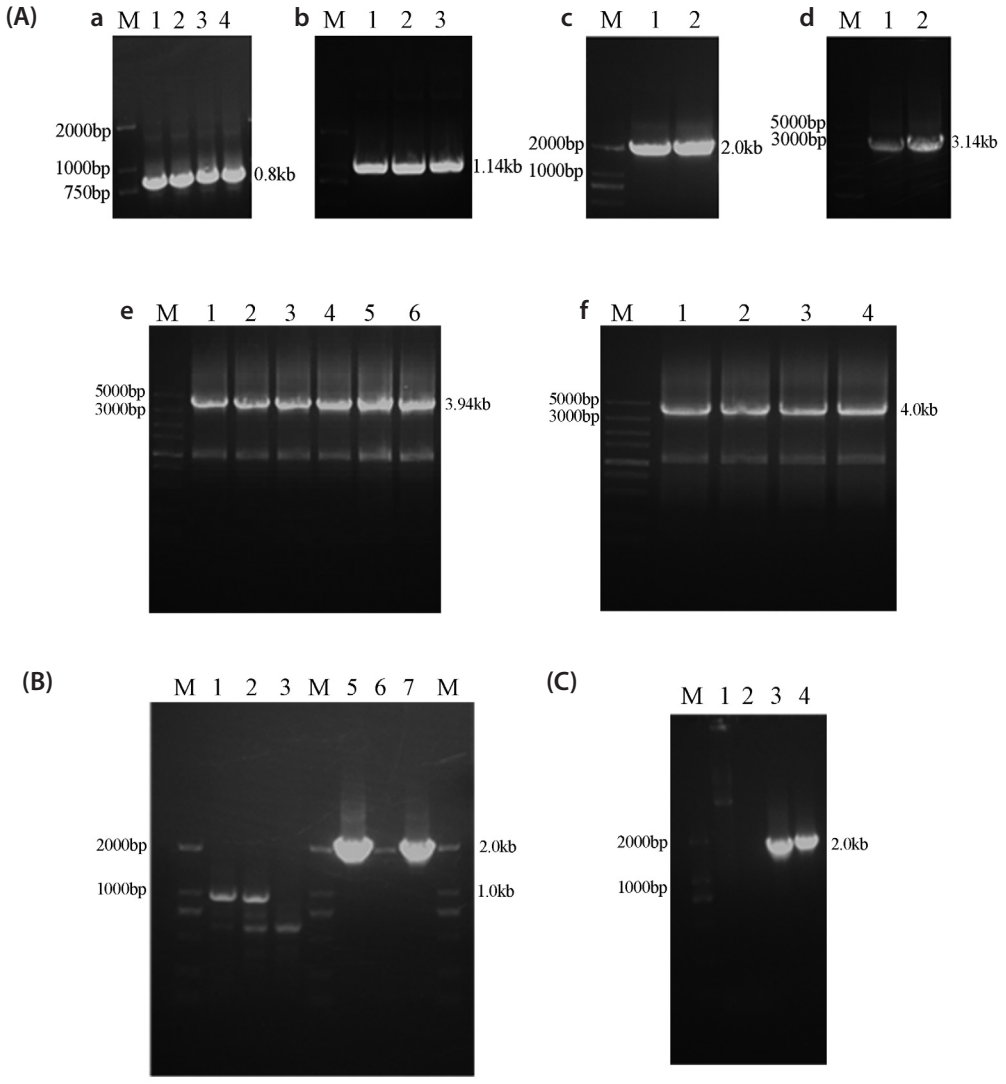

Fig. 2

Construction of the UL18-deletion BAC-HSV-1 plasmid

(A) The UL18 homologous recombination fragment (A-C-Kan^r-SacB-B) was constructed by PCR and analyzed by 1% agarose gel electrophoresis: (a) line M: DL 2000; line 1–4: segment C, product size 0.8 kb; (b) line 1–3: segment Kan^r, product size 1.14 kb; (c) line 1–2: SacB, product size 2.0 kb; (d-f) line M: DL 5000; (d) line 1–2: segment Kan^r-SacB-B, product size 3.14 kb; (e) line 1–6: C-Kan^r-SacB-B, product size 3.94 kb; (f) line 1–4: full deletion cassette, product size 4.0 kb. (B-C) The Kan^r and SacB marking genes were removed by the homologous recombination induced by rhamnose: line M: DL 2000; **(B)** line 1–3: UL18, product size 1.0 kb; line 5–7: SacB, product size 2.0 kb; **(C)** line 1–4: SacB, product size 2.0 kb.

amplified the downstream target gene set by PCR first, with a product size of approximately 0.8 kb, which was ligated to a roughly 50 bp section upstream of the *UL18* gene and called A-C fragment (Fig. 2Aa). Likewise, PCR amplification of kanamycin-resistant gene fragments as a forward screening maker gene (Kan^r) (Fig. 2Ab) and the levansucrase-coding gene as a reverse screening marker (SacB-B) was performed, and they were ligated to a roughly 50 bp terminal fragment of *UL18* (Fig. 2Ac). Using overlapping PCR twice and ligating Kan^r and SacB-B (Kan^r-SacB-B) as well as Kan^r-SacB-B (Fig. 2Ad-e) and A-C successively (Fig. 2Af), we ultimately obtained the UL18 homologous recombination fragment (A-C-Kan^r-SacB-B, product size approximately 4.0 kb).

Next, using homologous recombination, the A-C-Kan^r-SacB-B fragment was inserted into the correct site of BAC-HSV-1. A-C-Kan^r-SacB-B fragments were electroporated into electrocompetent recipient cells with BAC-HSV-1 and arabinose-inducible λ-Red recombinase expression. The electrocompetent recipient cells were incubated in SOC culture at 37°C for 1–2 h. The forward screening maker gene, Kan^r, was used to screen for bacteria with successful recombination, where the *UL18* gene was replaced by the marker genes. Then, we validated the monoclone by PCR amplification of the *UL18* gene and two marker genes, which showed that the positive control DNA (BAC-HSV-1) amplified the *UL18* gene, unlike the selected monoclone and negative control DNA (BAC) that could only amplify the Kan^r and SacB gene products (Fig. 2B-C). Thus, the Kan^r- SacB gene fragment had been recombined into the correct site to replace the *UL18* gene.

The second step of the homologous recombination was to remove the forward and reverse screening marker genes including Kan^r and SacB to eliminate the potential negative influence of the marker genes on viral proliferation. First, rhamnose was added to the LB medium to induce expression of I-SecI endonuclease in pREDI; I-SecI endonuclease cut the I-SecI recognition site between the Kan^r and SacB genes, which caused DNA breakage damage. The DNA repair system in the host identified the double-stranded DNA breakage, and then induced nearby homologous fragments to recombine for repair. The homologous recombination occurred between the C of A-C-Kan^r-SacB-B and the gene downstream of *UL18*; thus, the full-length *UL18* gene was deleted without any marker genes. Lastly, bacteria with successfully removed marker genes were selected by the SacB reverse maker gene coding levansucrase, which would result in bacterial death by catalyzing saccharose into fructose.

BAC-HSV-1ΔUL18 failed to achieve viral rescue

We generated a *UL18* knockout HSV-1 plasmid (BAC-HSV-1Δ*UL18*) by two-step homologous recombination *in vitro*. Next, we transfected the BAC-HSV-1ΔUL18 plasmid into Vero cells to obtain gene-deleted virus. The negative control (Vero+PBS) group showed no plaque formation or capsid protein VP5 expression. The positive control (BAC-HSV-1) group showed typical pathology (Fig. 3), with evident plaques (area enclosed by circular dotted box) and VP5 expression (area pointed by white arrow), verifying that the plaques were caused by virus infection. In contrast, the gene-knockdown (BAC-ΔUL18) group expressed VP5 but no viral plaque formation was observed (Fig. 3). We conferred that the progeny mutant virus cannot assemble normally when *UL18* is missing; thus, Vero cells did not form plaques. In order to further verify this mutant, we co-transfected BAC-ΔUL18 and pcDNA3.1-UL18 plasmids into Vero cells, which showed evident pathological reactions and VP5 expression (Fig. 3). This suggested that UL18 mutant HSV-1 is unable to package a regular capsid and is thus unable to produce a mature virion.

BAC-HSV-1ΔUL18 plasmid vaccine enhances cellular immune responses in mice dramatically

We have constructed a novel attenuated HSV-1 DNA vaccine above, a deletion mutant viral vector, which required assessment of immune effects by animal trials before being applied clinically. Using the BAC-HSV-1 plasmid as a positive control and the BAC plasmid as a negative control to assess the immunogenicity of the BAC-HSV-1ΔUL18 plasmid, the former produced infectious live virus in cells, unlike the latter.

Determination of serum specific antibody IgG (Table S1) and antibody neutralization assay (Table S2) were used to evaluate the humoral immune responses. Serum specific antibody IgG in vaccinated mice was determined by ELISA. The *N* value of the negative groups of mice was 0.167 >0.1, and ratios (*P*/*N*) of vaccinated groups and negative groups were <2.1, which showed that no significant difference between the two groups. Neutralization antibody assay indicated vaccinated groups of mice produced low antibody level, with a weak neutralization antibody ability. All in all, BAC-HSV-1ΔUL18 plasmid vaccine was on a low level of humoral immune responses stimulation.

To test the vaccine DTH of BAC-HSV-1ΔUL18, we injected 10 μl of inactivated HSV-1 suspension to the right subcutaneous auricle and 10 μl supernatant of the frozen lysate to the left auricle for mice in each group following the final immunization and measured the thickness of auricles 48 h later. The thickness of auricles represented the activity of T-lymphocytes. Results showed that the thickness of auricles in BAC-HSV-1ΔUL18-vaccinated mice had varied slightly but did not show any significant difference compared with the negative control (Fig. 4A). This indicated that the BAC-HSV-1ΔUL18 vaccine was safe and caused no clear hypersensitivity *in vivo*.

Further experiments evaluated cell immune responses. Spleens were isolated and extracted, and the splenocytes were used for T-lymphocytes proliferation and cytokines detection. BAC-HSV-1ΔUL18- and BAC-HSV-1-vaccinated

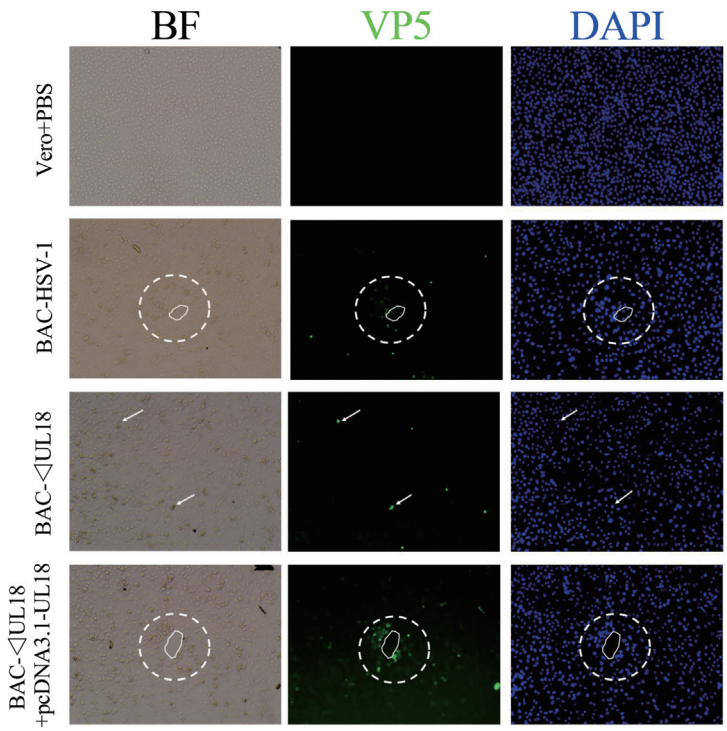

Fig. 3

Pathogenic detection of UL18 knockout mutant virus BAC-ΔUL18

Vero cells were seeded in 6 well culture plates and grown to 70% confluence for the different BAC plasmids as indicated in the figure transfection. After the positive control (BAC-HSV-1- transfected group) showed cytopathic effect, the cells were stained with VP5 antibody and DAPI. The area enclosed by the white circular dotted box indicates the plaque and the area indicated by white arrows shows VP5 expression. The fluorescence images were acquired using an OLYMPUS fluorescence microscope. BF: bright field.

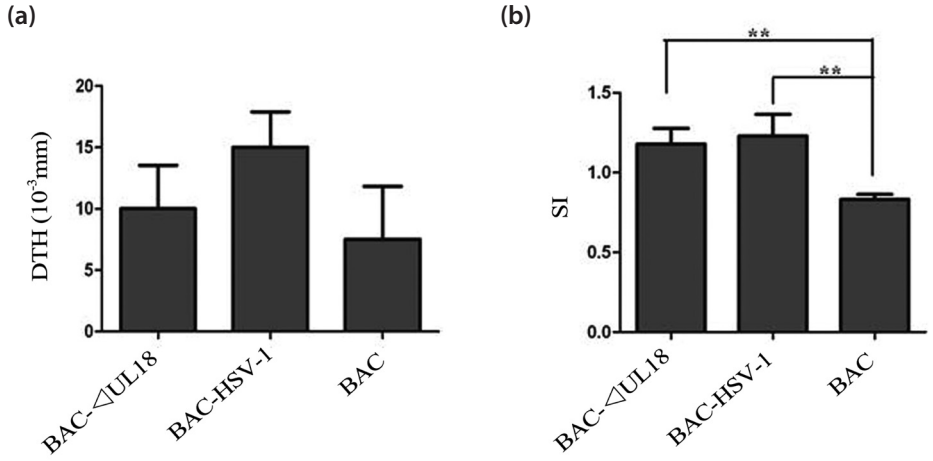

Fig. 4

Cellular immune responses in mice immunized with DNA vaccines

(a) The supernatants of plasmid BAC-ΔUL18, BAC-HSV-1 and BAC, respectively, were injected into the right subcutaneous auricle, meanwhile inactivated HSV-1 suspension was injected into the left. The thickness of auricles was measured 48 h later using a vernier caliper. DTH is calculated by subtracting the measurement for the left subcutaneous auricle from the right one; **(b)** Proliferation of splenocytes from the immunized and control groups of mice was measured by the MTT cell proliferation assay. Data are represented as stimulation index (SI), which is calculated by dividing the absorbance (OD) observed in experimental group by OD observed in negative cells.

groups showed specific T-lymphocyte proliferation responses, which significantly differed from those of the negative control (Fig. 4B). Cytokine detection was determined by ELISA. The results showed that IFN-γ secretion levels of the BAC-HSV-1ΔUL18 and BAC-HSV-1 vaccinated groups were significantly higher than those of the BAC group, while IL-2, IL-4, and IL-10 were not significantly different between BAC-HSV-1ΔUL18 and BAC vaccinated groups (Supplementary Table 1). In summary, the BAC-HSV-1ΔUL18 vaccine has the potential to enhance the cell immune responses *in vivo*.

Discussion

Homologous recombination is one of the most common methods applied to gene knockdown. The bacterial markerless gene knockdown system constructed by our laboratory has many advantages compared to conventional virus knockdown methods: (i) it is very efficient and can accomplish gene knockdown within 3 weeks; (ii) the full-length target gene can be deleted without any other exogenous genes, avoiding the negative influences from exogenous marker genes; (iii) extracorporeal operation simplifies the flow; (iv) the process of gene knockdown is relative safe since there is no contact with live virus; and (v) the system could, in theory, be used in multiple gene knockdown or insertion. Thus, the bacterial markerless gene knockdown system is bound to have extensive application such as in the construction of multiple gene knockdown vaccines and the functional study of viral genes.

As of yet, no HSV vaccine has been effective in humans, although most such vaccines play a part in animal trials for stimulating inspiring protective immunity. What hinders the smooth development of HSV vaccines is the complexity of the HSV life cycle and immune mechanism and its latent character. Researchers studying HSV mostly conduct clinical evaluations of subunit, attenuated live virus, defective virus, and naked DNA vaccines. DNA vaccines, as the most novel vaccines, rely on a strategy to guide the recombinant eukaryotic expression vector into an organism and then stimulate protective immune responses by transcription and translation in the host (Pasette *et al.*, 2003). DNA vaccines have many advantages compared with previous vaccines, including the impossibility of viral virulence resumption, persistent antigen expression, and low cost. In addition, the immune principle of DNA vaccines is similar to that of the natural viral infection.

BAC-HSV-1ΔUL18 is a type of DNA vector vaccine, where the *UL18* gene has been deleted through genetic engineering. Gene-deleted vaccines maintain the character of attenuated live virus vaccines, while preserving immunogenicity. The earliest mutant gene of HSV-1 was *TK* (Gordon *et al.*, 1987), a primary HSV virulence gene, but its mutant attenuated vaccine has shown different immune protective efficacy in different species. The *UL18* gene, as a component of the tegument, is an essential virulent gene of HSV-1, whose gene product VP23 stabilizes the structure of the tegument with VP5 and VP19C (Trus *et al.*, 1996); thus, a UL18-deficient virus is not able to assemble into a live infectious virus. The virus rescue experiment showed that Vero cells did not grow progeny mutant virus when infected with the BAC-HSV-1ΔUL18 plasmid but were able to do so when infected with BAC-HSV-1. Additionally, BAC-HSV-1ΔUL18 preserves other viral genes, including immunogenic components and glycoproteins.

The most important factor of HSV-1 in establishing lifelong infection is the character of immunologic escape. It is highly likely that DNA vaccines induce lifelong immunity and are involved in viral latency of the virus through cellular immunity, bearing both properties of prevention and therapy. Nevertheless, most non-replicating conventional vaccines cannot induce an effective cellular immune level. Lymphocyte proliferation is the most direct index of cellular immune level. The spleen, as the biggest immune organ, accounts for 25% of total lymphoid tissue. The cell proliferation assay verified that BAC-HSV-1ΔUL18 could significantly stimulate lymphocyte proliferation and then induce an effective protective immune response in mice.

Cytokines detection indicates that BAC-HSV-1ΔUL18 is able to improve the expression of IFN-γ that induces the virus clearing response of the TH1 helper T cells. IFN-γ and IL-4 represent a subset of TH1 and TH2 cytokines (Del Vecchio *et al.*, 2007). IFN-γ, which has many important immune functions, significantly enhances the MHC-II expression of antigen presenting cells (APC) and strengthens the interaction of APC and T cells; therefore, it strengthens the production of antibodies and cytotoxic lymphocytes (CTL) (Ferris *et al.*, 2006). In addition, IFN-γ can suppress viral replication by inducing the infected cells to produce various anti-virus proteins. IFN-γ is the activator of mononuclear macrophages, and strengthens the cytotoxicity of NK cells. Overall, IFN-γ plays a crucial role in cellular immunity. However, BAC-HSV-1ΔUL18 cannot stimulate expression of other cytokines including that of IL-2, IL-4 and IL-10. Improving the immunogenicity of BAC-HSV-1ΔUL18 requires the participation of other cytokines. At the same time, BAC-HSV-1ΔUL18 does not appear to induce hypersensitivity, which may induce autoimmune diseases.

Furthermore, BAC-HSV-1ΔUL18 cannot provide effective humoral immune responses in mice. The factors of determining the effectiveness of DNA vaccines depend on the choice of the target gene, the choice of vector and promoter, immunologic adjuvant, inoculation route, dose, and the organism itself. The development of the BAC-HSV-1ΔUL18 vaccine requires further study to improve its humoral immune responses, like most other HSV-1 vaccines. Neverthe-

less, there exists the potential significance of utilizing UL18 knockout in a HSV-1 DNA vaccine, which can be combined with the attenuated live virus vaccine by using the markerless gene knockout system *in vitro*.

Conclusions

In our study, combination of the pREDI plasmid and BAC-HSV-1 plasmid provides an effective bacterial markerless gene knockout system. Using the gene knockout system we successfully constructed a full-length UL18 gene knockout HSV-1 plasmid (BAC-HSV-1ΔUL18) by two-step homologous recombination. BAC-HSV-1ΔUL18 induces great cellular immune effect in mice and indicates the potential of exploiting UL18 knockout HSV-1 as a DNA vaccine.

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Supplementary information is available in the online version of the paper.

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Supplementary information

Construction, identification, and immunogenic assessments of an HSV-1 mutant vaccine with a *UL18* deletion

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Table S1. IgG of BAC-ΔUL18-, BAC-HSV-1- and BAC-vaccinated sera, respectively, and pre-immune sera as negative control were detected by ELISA

Groups / Serum dilution	BAC-HSV-1ΔUL18 (P/N±SD)	BAC-HSV-1 (P/N±SD)	BAC (P/N±SD)
1:400	1.218±0.032	1.170±0.027	1.135±0.016
1:800	1.178±0.029	1.087±0.030	0.893±0.017
1:1600	1.104±0.021	1.363±0.119	0.891±0.019
1:3200	1.209±0.043	1.078±0.019	0.905±0.014
1:6400	1.068±0.010	1.183±0.014	0.882±0.010
1:12800	1.023±0.007	1.090±0.027	0.926±0.015
1:25600	1.097±0.014	1.064±0.023	0.962±0.017

The antibody titers were determined as log of the reciprocal value of the highest serum dilution resulting in OD_{492} <0.1 and P/N ≥2.1 (*P* refers to the value of vaccinated mice OD; *N* refers to the value of negative control mice OD).

Table S2. BAC-ΔUL18-, BAC-HSV-1- or BAC-vaccinated sera were mixed with HSV-1 virus (100 TCID50) by double dilution and then incubated with Vero cells

Groups / Serum dilution	BAC-HSV-1 ΔUL18	BAC-HSV-1	BAC	No-Treated
100	++++	++++	++++	++++
200	++++	++++	++++	++++
400	++++	++++	++++	++++
800	++++	++++	++++	++++
1600	++++	++++	++++	++++
3200	++++	++++	++++	++++
6400	++++	++++	++++	++++
HSV-1 control	++++	++++	++++	++++
Cell control	-	-	-	-

Plus sign (+) represents positive cytopathic effect (CPE) and minus sign (-) represents negative of CPE. The infected Vero cells showed 75%-100% CPE, which was represented by "++++".