Antiviral Research 80 (2008) 272–279

Contents lists available at ScienceDirect

Antiviral Research

journal homepage: www.elsevier.com/locate/antiviral

A novel recombinant bacterial vaccine strain expressing dual viral antigens induces multiple immune responses to the Gag and gp120 proteins of HIV-1 in immunized mice

Yong Feng[a,1], Shiqun Wang[a,1], Fenglin Luo[b], Ying Ruan[a], Lei Kang[a], Xiaohui Xiang[a], Tao Chao[a], Guiqing Peng[a], Chengliang Zhu[a], Yongxin Mu[a], Ying Zhu[a], Xiaolian Zhang[b], Jianguo Wu[a,*]

[a] State Key Laboratory of Virology and College of Life Sciences, Wuhan University, Wychang, Wuhan 430072, Hubei, PR China
[b] State Key Laboratory of Virology and College of Medicine, Wuhan University, Wuhan 430071, PR China

ARTICLE INFO

Article history:
Received 24 April 2008
Received in revised form 15 June 2008
Accepted 16 June 2008

Keywords:
Vaccine
HIV
AIDS
Recombinant *Salmonella enterica serovar* Typhi vaccine strain
Immune responses
Cytotoxic T lymphocyte response
Antibody

ABSTRACT

Recombinant *Salmonella enterica serovar* Typhi can function as a live vector to deliver foreign antigens to the mammalian immune system and induce both mucosal and systemic immunity. In this study, we generated a recombinant *Salmonella* Typhi strain *pilS*$^-$*pilT*$^-$Gag$^+$(pVAX1-gp120) harboring the human immunodeficiency virus (HIV) *gag* gene integrated into the bacterial chromosome and *gp120* gene carried by a plasmid. Mice inoculated with this recombinant bacterium through intranasal route produced high titers of IgG to gp120 in sera and IgA to gp120 in fecal washes. In addition, Gag-specific and gp120-specific cytotoxic T lymphocyte (CTL) responses were observed in sorted spleen lymphocytes of immunized mice. These results demonstrated that this recombinant *Salmonella* Typhi strain elicits multiple immune responses against both Gag and gp120 antigens of HIV, and thus would be a potential vaccine candidate to the prevention of HIV/AIDS.

1. Introduction

The global dissemination of human immunodeficiency virus (HIV) and the fatal clinical results of acquired immunodeficiency syndrome (AIDS) urge scientists and physicians to discover the most potent prophylactic and therapeutic vaccines to combat this deadliest scourge of human society. Live recombinant microorganisms have been used as vectors to develop vaccines for eliciting broad array of immune responses against HIV/AIDS. Antigens of HIV can be brought into live and replication competent or incompetent microorganisms by molecular technologies. Appropriate expression of such antigens can be achieved under suitable conditions. When these recombinant vaccines are introduced to mammalian host, multiple immune responses, especially cytotoxic T lymphocytes (CTLs) activity will be elicited against the products of HIV genes carried by the vectors. A number of live viral vectors including poxvirus (Ondondo et al., 2006), alphavirus (Perri et al., 2003), adenovirus (Vinner et al., 2006), adeno-associated virus (Tatalick et al., 2005), rabies virus (Tan et al., 2005), measles virus (Lorin et al., 2004), and vesicular stomatitis virus (Egan et al., 2004) have been used as potential vectors for the development of HIV vaccines. Early-phase clinical trials of some of these viral vector vaccines have performed in humans. In addition, recombinant bacterial vectors, such as *Listeria monocytogenes* (Rayevskaya et al., 2003), *Shigella* (Xu et al., 2003), *Mycobacterium bovis* BCG (Kawahara et al., 2002), and *Salmonella* (Kotton et al., 2006) have also been investigated as potential vectors for the delivery of HIV antigens. Since bacterial live vectors can be produced with less cost and stored via lyophilization, they offer many advantages over viral vectors.

Recombinant *Salmonella enterica serovar* Typhi holds a prominent position among bacterial live vectors. Genetic backgrounds of this bacterium and types of virulence mutations have been well characterized. Recombinant *Salmonella* Typhi vaccines are quite easy to massively produce and store, which make these vaccinations economically more feasible and accessible. Recombinant *S.* Typhi has been used as oral typhoid vaccines and as live vector to deliver heterologous antigens that can stimulate mucosal, humoral, and cellular immune responses after immunizing animals via mucosal surfaces (Kotton, 2004). Foreign antigens can be stably

* Corresponding author. Tel.: +86 27 68754979; fax: +86 27 68754592.
E-mail addresses: jwu@whu.edu.cn, wu9988@vip.sina.com (J. Wu).
[1] These authors contributed equally to this work.

0166-3542/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.antiviral.2008.06.013

Table 1
Recombinant *Salmonella* strains constructed and used in this study

Strains	Descriptions	Culture conditions[a]
S. Typhi J341	*S.* Typhi wild type Ty2 Vi$^-$	LB
S. Typhi ST011	*S.* Typhi *pilS*$^-$*pilT*$^-$(pVAX1)	LB Km^R Cm^R
S. Typhi ST012	*S.* Typhi *pilS*$^-$ *pilT*$^-$(pVAX1-gp120)	LB Km^R Cm^R
S. Typhi ST013	*S.* Typhi *pilS*$^-$ *pilT*$^-$Gag$^+$(pVAX1)	LB Km^R Cm^R
S. Typhi ST014	*S.* Typhi *pilS*$^-$ *pilT* Gag$^+$(pVAX1-gp120)	LB Km^R Cm^R

[a] LB, Luria-Bertani broth; Km^R, kanamycin resistance; Cm^R, chloramphenicol resistance.

expressed in the bacteria either from its chromosome harboring the antigen gene or from a plasmid carrying the gene (Stephens et al., 2006). We have previously constructed a *S. enterica serovar* Typhi strain carrying the nucleocapsid (N) gene of severe acute respiratory syndrome-associated coronavirus (SARS-CoV) integrated into the bacterial chromosome, which could elicit efficiently immune responses in vaccinated mice (Luo et al., 2007).

In this study, we generated a new recombinant *S.* Typhi vaccine candidate *pilS*$^-$*pilT*$^-$Gag$^+$ (pVAX1-gp120) by a two-step genetic construction process. This vaccine strain harbors the *gag* gene of HIV-1 integrated into the bacterial chromosome and the *gp120* gene of HIV-1 carried by a eukaryotic plasmid. Immune responses against the two major antigens of HIV-1 were investigated after mucosal immunization of mice with this newly developed vaccine strain. Potential use of this recombinant *S.* Typhi strain as a promising vaccine against HIV/AIDS was also discussed.

2. Materials and methods

2.1. Plasmids, Salmonella strains and culture conditions

Plasmid pNL4-3 containing the full-length HIV-1 proviral sequence was obtained from NIH AIDS Research & Reference Reagent Program. Plasmid pBluescript II SK (+) was used as the basis for the construction of shift plasmid. Plasmid pVAX-1 was used to express HIV-1 gp120 protein. Plasmid pDNR-LIB was the provider of the Cm^R (Chloramphenicol resistance) gene. Plasmid pCMV-Tag 2B was used to construct Gag and gp120 expression plasmids which were applied in the establishment of stable CT26 cell lines constitutively expressing Gag or gp120 antigens.

The parental *S.* Typhi strain J341 was taken from laboratory stock (College of Medicine, Wuhan University, Wuhan, China). The cell culture conditions of all the *Salmonella* strains reported in this paper are listed in Table 1.

2.2. Construction of shift plasmid pBRGagCmU

The most important approach used in the construction of our recombinant vaccine is homologous recombination. First, we constructed a new shift plasmid pBRCmU, which can shift a heterologous gene from the plasmid to the chromosome of *S.* Typhi J341 strain. In brief, *pil*R gene from the genome of *S.* Typhi J341 strain was PCR amplified with primers 5′-AGTGGTACCTTGCATGAGTCCTTTATACCG-3′ and 5′-TACGGGCCCTCCAGTTCGACAGGGACCACCTGGT-3′. The PCR product was then inserted into plasmid pBluescript II SK (+) at the KpnI and ApaI sites as the left arm of shift plasmid. *pil*U gene from the genome of *S.* Typhi J341 strain was PCR amplified with primers 5′-ATACCGCGGGCAAACGGGGGCTCAATGACAGTCT-3′ and 5′-TTAGAGCTCACCCCCATCCAGCCTACCAGGGCAC-3′. The PCR product was then inserted into plasmid pBluescript II SK (+) at the SacII and SacI sites as the right arm of shift plasmid. This new plasmid was named pBRU. The Cm^R (Chloramphenicol resistance) gene from plasmid pDNR-LIB was amplified by PCR with primers 5′-GGCTCTAGAAGGAAGCTAAAATGGAGAA-3′ and 5′-AAACCGCGGAAATTACGCCCCGCCCTGCC-3′ and then inserted into plasmid pBRU at the XbaI and SacII sites to generate shift plasmid pBRCmU.

The *gag* gene of HIV-1 was amplified from plasmid pNL4-3 by PCR with primers 5′-ATACTCGAGGAGATGGGTGCGAGAGCGT-3′ and 5′-GGCGAATTCATCTTTATTGTGACGA-3′ and then inserted into shift plasmid pBRCmU at XhoI and EcoRI sites to yield shift plasmid pBRGagCmU (Fig. 1).

2.3. Construction of recombinant S. enterica serovar Typhi strain S. Typhi pilS$^-$pilT$^-$Gag$^+$ strain stably expressing Gag antigen of HIV-1

Plasmid pBRGagCmU was transformed into *S. enterica serovar* Typhi J341 via the *S. enterica serovar* Typhimurium modifying strain J357 and with selection for Amp^R and Cm^R colonies. Individual clones were selected and grown in 5 ml LB media without antibiotic selection at 42 °C for 24 h. Then 0.05-ml aliquots were transferred into 5 ml fresh medium. After three to four transfers, aliquots were plated onto LB media containing Cm and the grown-up colonies that had already lost transformed plasmid pBRGagCmU were confirmed by checking for Amp sensitivity. Finally, the recombinant *S. enterica serovar* Typhi *pilS*$^-$*pilT*$^-$Gag$^+$ strain (ST014), in which the *gag* gene was integrated into the bacterial genome, were isolated from the above colonies by screening for both Cm resistance and Amp sensitivity. The integration of the *gag* gene was examined by PCR amplification using primers adjacent to the inserted fragment.

2.4. Western blot assay for Gag expression

The cultured recombinant *S.* Typhi strain *pilS*$^-$*pilT*$^-$Gag$^+$ was sonicated on ice and the cell lysate was electrophoresed on 12% SDS polyacrylamide gels and transferred overnight to a cellulose nitrate membrane. After transfer, the membrane was blocked in 5% skim milk powder/PBS containing 0.1% Tween (PBS-T) for overnight with shaking at 60 rpm. Then the membrane was washed three times with PBS-T for 10 min per wash and incubated for 1 h at RT

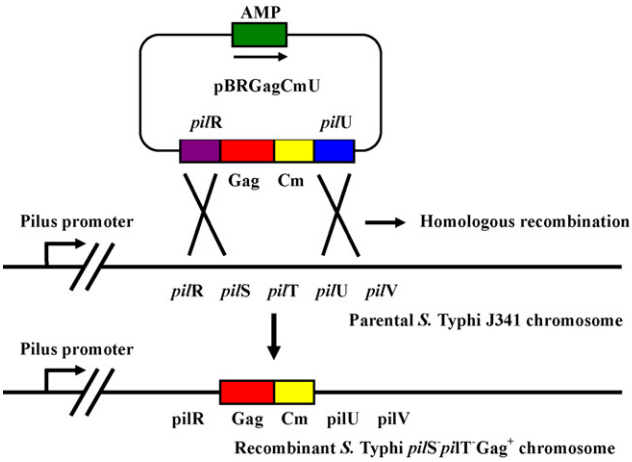

Fig. 1. Schematic diagram of the construction of recombinant *Salmonella enterica serovar* Typhi strain *S.* Typhi *pilS*$^-$*pilT*$^-$Gag$^+$. Plasmid pBRGagCmU (upper part) containing the gag gene of HIV-1 and the Cm^R gene flanked by the *pli*R and *pli*U genes of *S. enterica serovar* Typhi was transformed into *S. enterica serovar* Typhi J341 (middle part). The HIV-1 gag gene and Cm^R gene carried by the plasmid were then integrated into the *pil*R and *pil*U loci of *S. enterica serovar* Typhi J341 chromosome through homologous recombination chromosomal segments to generate recombinant *S. enterica serovar* Typhi strain *S.* Typhi *pilS*$^-$*pilT*$^-$Gag$^+$ (bottom part). In this recombinant strain the expression of HIV-1 gag gene was under the control of the pilus promoter of the bacterium.

with 1:1000 diluted mouse anti-P24 monoclonal antibodies. The cellulose nitrate membrane was washed three times with PBS-T and incubated with horseradish peroxidase (HRP)-labeled goat anti-mouse IgG (1:2000 diluted, Sigma–Aldrich, Germany) in PBS-T for 1 h at RT. The membrane was incubated with chromogen and exposed to X-ray film for 30 s and 1 min.

2.5. Construction of plasmid pVAX1-gp12

The *gp120* gene of HIV-1 was amplified from plasmid pNL4-3 using primers 5′-TCGGGATCCGTGGCAATGAGAGTGAAG-3′ and 5′-GCTGAATCCTCATCCTATTCCCACTGC-3′. The PCR product was then inserted into plasmid pVAX1 at the BamHI and EcoRI sites to generate a eukaryotic expression plasmid pVAX1-gp120.

2.6. Western blot assay for gp120 expression

The eukaryotic expression plasmid pVAX1-gp120 was transfected into 293T cells according to the standard cell transfection procedure. Transfected cells were harvested in 48 h and sonicated on ice subsequently. After centrifugation, the supernatant was used for western blot assay. Serum from an HIV-1 positive patient was used as the source of primary antibody (1:1000 diluted). The secondary antibody was horseradish peroxidase (HRP)-labeled goat anti-human IgG (1:2000 diluted, Sigma–Aldrich, Germany).

2.7. Construction of S. enterica serovar Typhi vaccine candidate strain S. Typhi $pilS^-pilT^-Gag^+$ (pVAX1-gp120)

Plasmid pVAX1-gp120 was modified by J357 and transformed into the recombinant strain *S.* Typhi $pilS^-pilT^-Gag^+$ to yield the strain *S.* Typhi $pilS^-pilT^-Gag^+$(pVAX1-gp120) (also named ST014) as the vaccine candidate strain.

2.8. Construction of recombinant Salmonella control strains

By using similar approaches, three different control recombinant strains were also constructed. In strain *S.* Typhi $pilS^-pilT^-$(pVAX1) (ST011), the shift plasmid pBRCmU carrying only the Cm^R gene was integrated into the bacterium J341 genome and plasmid pVAX1 was transformed into the bacterium subsequently. In strain *S.* Typhi $pilS^-pilT^-$(pVAX1-gp120) (ST012), the shift plasmid pBRCmU carrying only the Cm^R gene was integrated into the bacterium J341 genome and plasmid pVAX1-gp120 instead of pVAX1 was transformed into J341 compared to ST011. In strain *S.* Typhi $pilS^-pilT^-Gag^+$ (pVAX1) (ST013), the shift plasmid pBRGagCmU was integrated into the bacterium J341 genome and plasmid pVAX1 was transformed into J341 (Table 1).

2.9. Establishment of stable CT26 cell lines constitutively expressing Gag or gp120 antigens

The *gag* gene and *gp120* gene of HIV-1 were amplified by PCR from plasmid pNL4-3 using primers for Gag: 5′-GCAGGATCCATGGGTGCGAGAGCGTCGGT-3′ and 5′-ACGGAATTCTTATTGTGACGAGGGGTCGC-3′, and primers for gp120: 5′-TCGGGATCCGTGGCAATGAGAGTGAAG-3′ and 5′-GCTGAATCCTCATCCTATTCCCACTGC-3′. PCR products were then inserted, respectively at the BamHI and EcoRI sites of plasmid pCMV-Tag2B to generate the eukaryotic expression plasmids pCMV-Tag2B-Gag and pCMV-Tag2B-gp120. These two plasmids were then transfected into mouse CT26 cells. After G418 selection for 30 days, the antigen constitutively expressing cells were checked by RT-PCR and stored as targeted cells for cytotoxicity assay.

2.10. Immune responses of recombinant S. Typhi in mice

2.10.1. Mice immunization

Female BALB/c mice aged 6–8 weeks were randomly divided into five groups with five mice in each group. Group 1 was immunized with *S.* Typhi ST011, group 2 with *S.* Typhi ST012, group 3 with *S.* Typhi ST013, group 4 with *S.* Typhi ST014, and group 5 was PBS control group. Mice were immunized intranasally with 10^9 CFU of bacteria in 10 μl of PBS. The booster immunization with 10^9 CFU in 10 μl PBS was also given intranasally 15 days after first immunization.

2.10.2. ELISA for mouse serum anti-gp120 IgG and anti-Gag IgG

Immulon II microtiter plates coated with purified HIV-1 Env protein or purified recombinant p24 protein (Dynatech Laboratories, USA) were used in ELISA for detection of mouse serum anti-gp120 IgG and anti-Gag IgG. Plates were washed five times with PBS-T, blocked with 3% BSA in PBS-T at room temperature for 1 h prior to the addition of mouse sera. Sera were subjected for titer determination in eight twofold dilutions staring at 1:100 up to 1:12800. Antibodies bound to the immobilized protein antigens were detected using horseradish peroxidase-labeled goat anti-mouse IgG diluted 1:5000 in PBS-T containing 1% BSA and substrate solution containing *o*-phenylenediamine (1 mg/ml) and H_2O_2 (0.03%) in 0.1 M citrate-phosphate buffer. The reaction was stopped with 2 mM H_2SO_4. The absorbance was measured on a model 3550 Microplate Reader (Bio-Rad Laboratories, USA) at 450 nm. Test and control sera were run in duplicate.

2.10.3. ELISA for mouse mucosal anti-gp120 IgA

To detect the mucosal secreted IgA (S-IgA) level stimulated by the vaccine candidate, we harvested fecal, vaginal, and saliva samples from immunized mice. Immulon II microtiter plates coated with purified HIV-1 Env protein (Dynatech Laboratories, USA) were used in ELISA for the detection of anti-gp120 IgA in mouse samples. These mucosal samples were mixed with an equal volume of PBS and eight twofold dilutions of their supernatants were used for ELISA. Samples were added into microtiter plates at 100 μl per well and incubated at 37 °C for 1 h. Horseradish peroxidase-conjugated goat anti-mouse IgA antibodies were added at a 1:5000 dilution in PBS-T containing 1% BSA for incubation at 37 °C for 1 h. Test and control groups were run in duplicates. So the S-IgAs we detected were mucosal IgA, not plasma IgA.

2.10.4. Enumeration of HIV-1 Gag-specific and gp120-specific T cell cytotoxicity by Promega LDH release assay

Splenocytes from immunized BALB/c mice were suspended in complete RPMI-1640 with 10% FCS and analyzed for cytotoxic activity. Serial twofold dilutions of mice splenocytes as expanded effector cells (7.8×10^2 to 2×10^5 cells/wells) were incubated, respectively with target CT26 cells (2×10^4 cells/wells) that stably expressed Gag protein or target CT26 cells (2×10^4 cells/wells) that stably expressed gp120 protein. Cultures were centrifuged at $250 \times g$ for 4 min and incubated for 4 h at 37 °C in the presence of 5% CO_2. Fifty microliters of the supernatant per well were then transferred to enzymatic assay plates, and lysis was determined by measuring released lactate dehydrogenase (LDH) by using the Cytotox 96 assay kit (Promega Corp., Madison, WI, USA). The absorbance values from supernatants were recorded at 490 nm on a model 3550 Microplate Reader (Bio-Rad Laboratories, USA). Cultures were tested in triplicate wells. The percentage of cytotoxicity was calculated

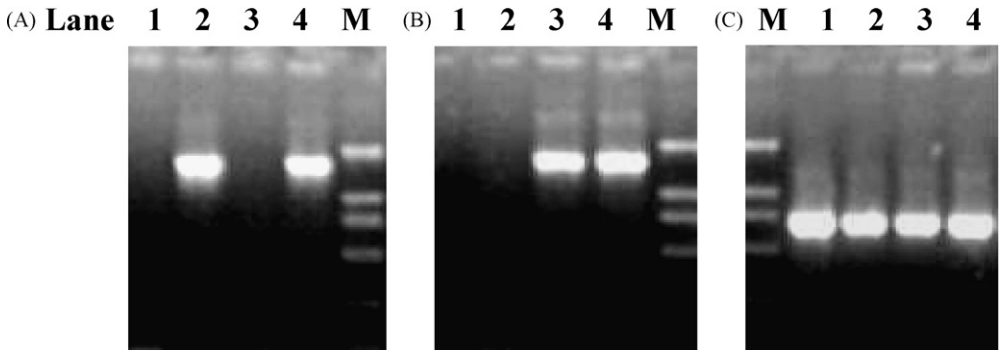

Fig. 2. Determination of foreign genes in bacterium by PCR analysis. The existing of the foreign genes, gp120 (Fig. 2A), gag (Fig. 2B), and Cm^R (Fig. 2C), in recombinant *Salmonella* strains, ST011 (lane 1), ST012 (lane 2), ST013 (lane 3), and ST014 (lane 4), were determined by PCR amplification using specific primers to each genes, respectively. M, DNA molecular markers DL2000.

as follows: (experimental – effector spontaneous – target spontaneous)/(target maximum – target spontaneous) × 100, where spontaneous release is the counts released by target cells in the absence of effector cells and maximal release is the counts released in the presence of lysis solution.

2.11. Statistical analysis

Data were expressed mean ± standard deviation (S.D.) and statistical analyses between groups were performed by one-way analysis of variance (ANOVA) and *q* test using the SPSS 10.0 software. Statistical significance was established at a value of $P < 0.05$.

3. Results

3.1. Recombinant Salmonella strains harboring the gag gene integrated into the bacterial chromosome and the gp120 gene carried by a plasmid are generated

To generate the vaccine candidate recombinant *Salmonella* strain *S.* Typhi *pilS*$^-$*pilT*$^-$Gag$^+$(pVAX1-gp120) (ST014), plasmid pBRGagCmU carrying the *gag* gene of HIV-1 and the antibiotic gene Cm^R flanked by the sequences of *Salmonella pil*R and *pil*U genes was initially transformed into *S. enterica serovar* Typhi J341. Recombination occurred between the plasmid DNA and *Salmonella* chromosome DNA through homologous recombination within *pil*R and *pil*U genes. The *gag* gene of HIV-1 and Cm^R gene were inserted into the chromosome in place of the *pilS* and *pilT* genes of *Salmonella* to generate recombinant strain *S.* Typhi *pilS*$^-$*pilT*$^-$Gag$^+$ (Fig. 1). Plasmid pVAX1-gp120 carrying the gp120 gene of HIV-1 was transformed into *pilS*$^-$*pilT*$^-$Gag$^+$ to yield strain *S.* Typhi *pilS*$^-$*pilT*$^-$Gag$^+$ (pVAX1-gp120) (named ST014). By using similar approaches, three different control recombinant strains ST011, ST012, and ST013 were also constructed as described in Section 2 and listed in Table 1.

3.2. The gag gene and gp120 gene of HIV-1 are expressed in recombinant Salmonella vaccine strains

The expression of HIV-1 *gag* gene is under the control of pilus promoter of the *Salmonella* chromosome and the expression of HIV-1 *gp120* gene is under the control of P_{CMV} promoter of plasmid pVAX1 in the vaccine strain ST014. The existing of the foreign genes in recombinant *Salmonella* strains were confirmed by PCR amplification using specific primers for *gp120* (Fig. 2A), *gag* (Fig. 2B), and Cm^R (Fig. 2C), respectively. PCR results showed that the *gp120* gene was present in strain ST012 (Fig. 2A, lane2) and ST014 (Fig. 2A, lane 4), but not in strain ST011 (Fig. 2A, lane 1) and ST013 (Fig. 2A, lane 3). The *gag* gene was detected in strain ST013 (Fig. 2B, lane 3) and ST014 (Fig. 2B, lane 4), but not in strain ST011 (Fig. 2B, lane 1) and ST012 (Fig. 2B, lane 2). The Cm^R gene was detected in all four recombinant *Salmonella* strains (Fig. 2C, lanes 1–4).

The status of gp120 protein and Gag protein expression was examined by Western blot analyses using antibody specific to gp120 protein (Fig. 3A) and Gag protein (Fig. 3B), respectively. Results from Western blot analyses indicated that the gp120 protein was not detected in 293T cells transfected with plasmid pVAX1 (Fig. 3A, lane 1), but expressed in 293T cells transfected with plasmid pVAX1-gp120 (Fig. 3A, lane 2). The Gag protein of HIV-1 was not detected in *Salmonella* strain J341 (Fig. 3B, lane 1), but expressed in strains ST013 (Fig. 3B, lane 2) and ST014 (Fig. 3B, lane 3).

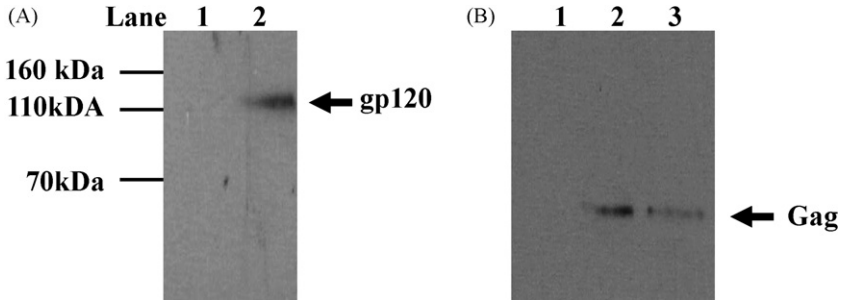

Fig. 3. Determination of viral protein expression in bacteria. Western blot assay for gp120 and Gag expression by Western blot analysis. (A). Determination of HIV-1 gp120 protein expression in 293T cells. 293T cells were transformed with plasmid pVAX1 (lane 1) and pVAX1-gp120 (lane 2). Proteins expressed in the mammalian cells were determined by Western blot analysis using antibody to HIV-1 gp120. (B) Determination of HIV-1 gag protein expression in recombinant *Salmonella* vaccine strains. Protein extracts were prepared from *Salmonella* strains, *S.* Typhi J341 (lane 1), ST013 (lane 2), and ST014 (lane 3), respectively. Proteins expressed in the bacteria were determined by western blot analysis using antibody to HIV-1 Gag protein.

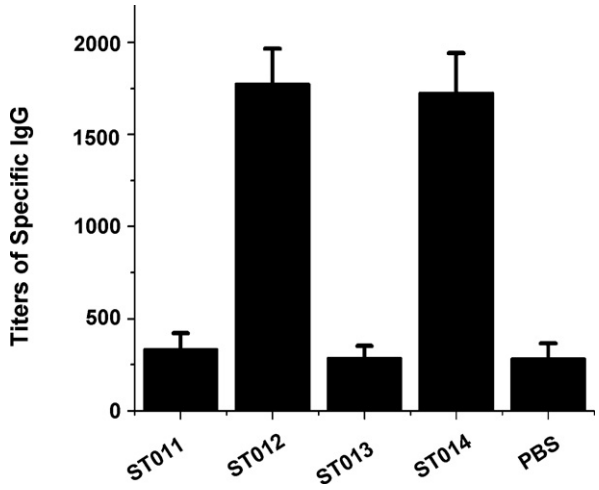

Fig. 4. Detection of serum anti-HIV gp120 IgG production in mice immunized with recombinant *S.* Typhi vaccine strains by ELISA. Five groups of BALB/c mice were inoculated i.n. twice with 10^9 CFU of recombinant *S.* Typhi vaccine strains ST011, ST012, ST013, ST014, and PBS, respectively. Two weeks post-immunization, sera were collected from vaccinated mice and analyzed by ELISA using purified HIV-1 Env protein as antigen for the detection of gp120-specific IgG antibodies responses. Horseradish peroxidase-conjugated goat anti-mouse IgG antibodies were used as the secondary antibodies. The optical densities were measured at 450 nm. Results represent the values (mean ± S.D.) for five mice per group from three different experiments.

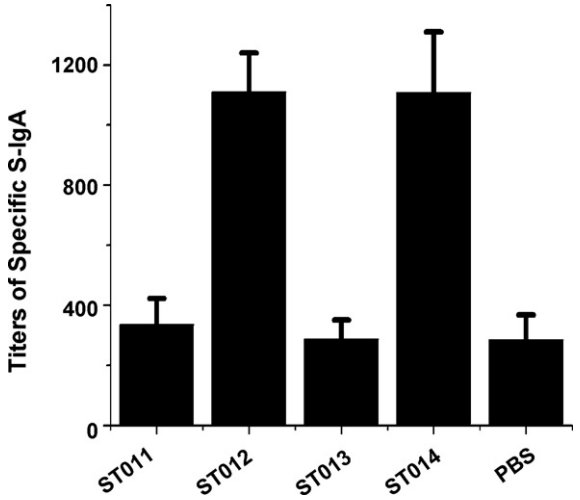

Fig. 5. Detection of intestine mucosal anti-HIV gp120 IgA production in mice immunized with recombinant *S.* Typhi vaccine strains by ELISA. Five groups of BALB/c mice were inoculated i.n. twice with 10^9 CFU of recombinant *S.* Typhi vaccine strains ST011, ST012, ST013, ST014, and PBS, respectively. Two weeks post-immunization, fecal washes were collected from vaccinated mice and analyzed by ELISA using purified HIV-1 Env protein as antigen for the detection of gp120-specific IgA antibodies responses. Horseradish peroxidase-conjugated goat anti-mouse IgA antibodies were used as the secondary antibodies. The optical densities were measured at 450 nm. Results represent the values (mean ± S.D.) for five mice per group from three different experiments.

3.3. Recombinant Salmonella vaccine strains induce high IgG antibody responses to gp120 protein of HIV-1 in immunized mice

To determine immune responses of recombinant *Salmonella* strains, BALB/c mice were randomly divided into five groups and vaccinated intranasally with 10^9 CFU of the recombinant bacteria, respectively. Group 1 was immunized with strain ST011, group 2 with ST012, group 3 with ST013, group 4 with ST014, and group 5 was inoculated with PBS as a mock control. The booster immunization with 10^9 CFU of recombinant bacteria was also given intranasally 15 days after first immunization.

Two weeks post-immunization, the gp120-specific IgG antibodies in the sera of immunized mice were determined by ELISA using purified HIV-1 Env protein as antigen. Results showed that high levels of gp120-specific IgG antibody responses in sera of mice vaccinated with ST012 or ST014 were detected compared to that of other three groups (Fig. 4). The production of anti-Gag-specific antibodies in the serum of immunized mice were also determined by ELISA using p24 protein of HIV-1 as antigen. However, results showed that antibodies against p24 protein were not detected in sera after immunization with these recombinant *Salmonella* vaccine strains (data not shown).

3.4. Immunization with recombinant Salmonella vaccine strains stimulate high IgA antibody responses to gp120 protein of HIV-1 in the feces of vaccinated mice

To determine IgA antibody responses of vaccine strains, mice were vaccinated intranasally with 10^9 CFU of the recombinant bacteria. The booster immunization with 10^9 CFU of recombinant bacteria was given intranasally 15 days after first immunization. IgA antibody responses to gp120 protein from feces, vaginal secretions, and saliva of immunized mice were analyzed. Results from ELISA showed that high levels of gp120-specific IgA antibody activities were found only in feces of mice vaccinated with strains ST012 and ST014, but not in mice immunized with strains ST011, ST013, or PBS (Fig. 5). We also noticed that gp120-specific IgA antibody activities were not detected in vaginal secretions or saliva of mice immunized with all the four vaccine strains (data not shown).

3.5. Immunization with recombinant Salmonella vaccine strains elicit HIV-1 gp120-specific cytotoxic T lymphocytes responses in vaccinated mice

To analyze whether recombinant *Salmonella* vaccine strains could induce HIV-1 gp120-specific CTL responses, vaccinated mice were sacrificed 3 weeks post-immunization. Splenocytes were harvested and the CTL activities were measured by the Promega LDH

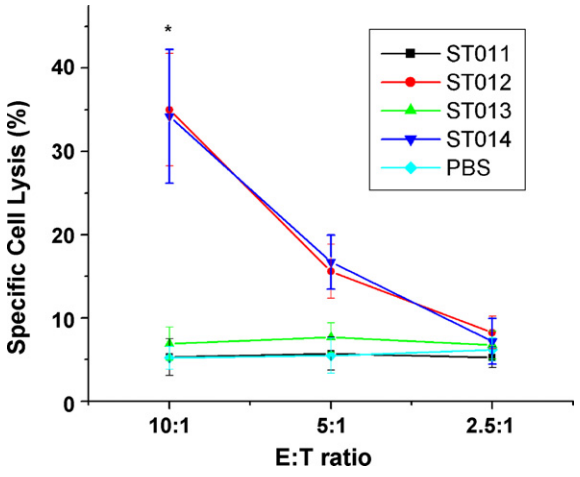

Fig. 6. Determination of gp120-specific CTL activity in mice immunized with recombinant *S.* Typhi vaccine strains. Five groups of BALB/c mice were inoculated i.n. twice with 10^9 CFU of recombinant *S.* Typhi vaccine strains ST011, ST012, ST013, ST014, and PBS, respectively. Three weeks post-immunization, mice were sacrificed and CTL activities were measured by the Promega LDH release assay. Mouse CT26 cells constitutively expressing HIV-1 gp120 were used as target cells and splenocytes from immunized mice were used as effector cells. Results represent the values (mean ± S.D.) for five mice per group from three different experiments.

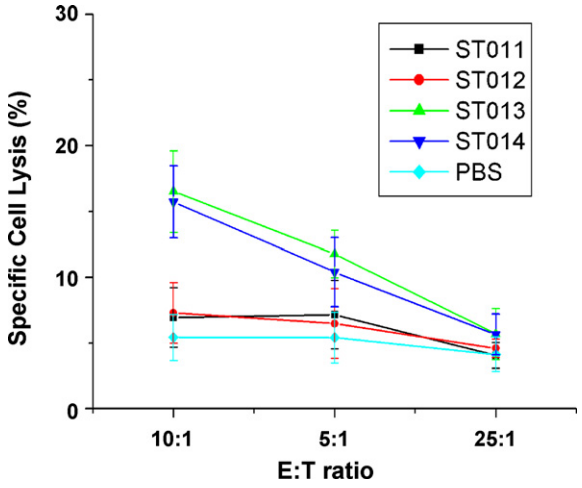

Fig. 7. Determination of Gag-specific CTL activity in mice immunized with recombinant *S.* Typhi vaccine strains. Five groups of BALB/c mice were inoculated i.n. twice with 10^9 CFU of recombinant *S.* Typhi vaccine strains ST011, ST012, ST013, ST014, and PBS, respectively. Three weeks post-immunization, mice were sacrificed and CTL activities were measured by the Promega LDH release assay. Mouse CT26 cells constitutively expressing HIV-1 Gag were used as target cells and splenocytes from immunized mice were used as effector cells. Results represent the values (mean ± S.D.) for five mice per group from three different experiments.

release assay. Results showed that strong CTL responses were produced in mice immunized with the vaccine strains ST012 and ST014, but not detected in mice vaccinated with strains ST011 and ST013 or in the mock controls (Fig. 6). When effecter to target ratio was 10:1, the gp120-specific CTL activity reached to 30%, while at 5:1 gp120-specific CTL activity reached above 15% in mice immunized with the vaccine strains ST012 and ST014.

3.6. *Immunization with recombinant Salmonella vaccine strains elicit HIV-1 Gag-specific cytotoxic T lymphocytes responses in vaccinated mice*

HIV-1 Gag-specific CTL responses induced by recombinant *Salmonella* vaccine strains were also determined. Mice were sacrificed 3 weeks after immunization and CTL activities were measured by the Promega LDH release assay. Results showed that HIV-1 Gag-specific CTL responses were produced in mice vaccinated with strains ST013 and ST014 and that when effecter to target ratios were 10:1 and 5:1, Gag-specific CTL reached about 17% and 12%, respectively. However, HIV-1 Gag-specific CTL responses were not detected in mice immunized with strains ST011 and ST012 (Fig. 7).

4. Discussion

HIV is transmitted by both venereal and parental routes. An ideal preventive vaccine must accordingly elicit both mucosal immunity that blocks sexual transmission of the virus and systemic immunity that eliminates virus transmitted hematogenously. Foreign antigen expression, delivery, and presentation mechanisms, as well as the innate biological and genetic traits of *Salmonella* vectors have been intensively investigated, all demonstrated that this living bacterium is a strong candidate for HIV vaccine development. Many studies have reported that attenuated derivatives of *S. enterica serovar* Typhi carrying HIV antigens could stimulate immune defenses in mammalians (Devico et al., 2002; Fouts et al., 1995; Hone et al., 1994).

Because HIV exists as cell-associated or cell-free particles in infected host, a fully effective vaccine should induce both cell mediated immune (CMI) responses that damage virus infected cells and neutralizing antibodies that target viral particles directly. CMI has proven to be critical for effective early control of replicating virus and clearance of intracellular pathogens. It is widely accepted that CMI against internal viral proteins of HIV is essential for eliminating infected cells because these viral proteins are structurally important and rarely generate mutant epitopes (Sacha et al., 2007). To induce durable and effective CMI responses, these viral proteins have to be endogenously synthesized to ensure their entering into proper antigen presentation pathway. A recombinant non-pathogenic *Leishmania* vaccine harboring an HIV-1 Gag expressing plasmid was reported to elicit cell mediated immunity (Breton et al., 2007).

We generated a recombinant *Salmonella* vaccine strain ST014, in which HIV-1 *gag* gene was integrated into the bacterial chromosome and HIV-1 *gp120* gene was carried by a plasmid. Chromosomal integration generally increases the stability of foreign antigens and protects them from degradation by bacterial proteases (Kotton, 2004; Williams and Szalay, 1983). The Gag antigen expression (Fig. 3B) and Gag-specific CTL activity (one of the most important CMI responses) (Fig. 7) were detected in mice immunized with this vaccine strain. Cellular immunity may prevent virus from attacking new susceptible cells, while antibodies play important roles in the first line of defense. As for the development of HIV vaccines, the viral envelop glycoprotein gp120 has always been the immunogen of choice (Giri et al., 2004). Since the HIV gp120 is responsible for attachment to susceptible cells and is analogous to the highly immunogenic, its specific antibodies are considered to be crucial in the initial rounds of the campaign against HIV infection. In addition to CTL response, this recombinant vaccine strain could produce gp120 protein in mammalian cells (Fig. 3) and stimulate gp120-specific antibodies production in vaccinated mice (Figs. 4 and 5). There is evidence that multiple immune responses against multiple viral antigens (at least two) would establish superiority effects in the development of HIV vaccine (Amara and Robinson, 2002).

Although this recombinant *Salmonella* vaccine strain could not stimulate the production of Gag-specific antibodies in nasal immunized mice (date not shown), but could elicit a broad range of gp120-specific immune responses (Figs. 4–6). In addition, the gp120-specific CTL response was much higher than Gag-specific CTL response in the same experimental group (Figs. 6 and 7).

The gp120 glycoprotein complex enables the virus to attach to and fuse with the target cells to initiate the infectious cycle and is proved to be a highly immunogenic antigen. The Gag protein is an inner protein that helps assembly of viral nucleocapsid with less B cell epitopes and less immunogenic compared to gp120. Unlike the gp120 protein expressed from the eukaryotic plasmid, the level of Gag protein expressed from *S.* Typhi chromosome is relatively low when introduced into mammalian host. Thus, less anti-Gag antibodies (IgG or IgA) were produced in our study. This result was consistent with other report, which also showed that Gag-specific antibodies were hardly detected (Breton et al., 2007). However, since the sequences of Gag are more conservative than that of gp120, the cellular reaction against this important structural protein would be more crucial in the long-term host immune response against HIV.

We are currently in the process of addressing the above problem by utilization of appropriate and stronger promoters in *Salmonella* chromosome in order to optimize the chromosomal gene expression and antigen production to enhance the immune responses to the Gag antigen.

Our previous work has compared the efficiencies of nasal or oral routs of immunization in mice inoculated with recombinant *Salmonella* living vaccines and demonstrated that intranasal vaccination was more efficient than orogastric immunization (Luo et al., 2007). Thus, we chose intranasal immunization instead of

orogastric immunization for the induction of mucosal and systemic anti-HIV immune responses in this study. It is generally agreed that orogastric route for live bacterial vectors may be inefficient because the gastrointestinal tract contains commensal bacteria, other microorganisms, stomach acid, proteolytic enzymes, together with food might interfere with vaccine uptake and suppress immune responses (Galen et al., 1997; Pickett et al., 2000). In contrast, the nasal cavity surface provides a smooth environment that is replete with antigen-reactive lymphoid elements, including T cells, B cells, and a large number of cellular components involved in the induction of immune responses. Intranasal immunization could lead to the induction of distant mucosal immune responses (Ogra et al., 2001; Wu et al., 1997). The genital, rectal, and oral sites are considered to be most important for induction of strong mucosal immune responses to block the invasion of HIV through sexual intercourse.

gp120-specific IgA mucosal antibodies were detected in fecal washes (but not in saliva or vaginal secretions), which suggests that rectal immune response was elicited by the vaccine candidate. It has been reported that HIV infection rates were higher for homosexual injection drug users (34%) than for bisexuals (16%) or heterosexuals (13%) (Wolitski et al., 1992). Thus, we considered that rectal secreted S-IgA was important in the development of anti-HIV vaccine, since rectal sex is quite common in homosexual population.

In this study, we obtained genes from plasmid pNL4-3 containing the full-length HIV-1 proviral sequence, which is CXCR4 specific, while primary infection is sustained almost invariably by CCR5-dependent viruses. Thus, this represents a potential limitation of the possibility of translating the results to clinics. Experiments with an HIV-1 clone specific to CCR5 would be more suitable. However, the main purpose of this study was to test this vaccination strategy for the induction of broadly immune responses to multiple HIV antigens. On the basis of this platform we constructed, it is quite convenient to test antigens derived from other viral strains and evaluate the immune responses they might induce.

5. Conclusions

We generated a novel recombinant *S. enterica serovar* Typhi strain that can present the gag and gp120 dual antigens of HIV-1 to the host. Initial experimental results demonstrated that this *Salmonella* vaccine strain could elicit multiple immune responses, including humoral, cellular, and mucosal responses, in mice immunized intranasally with this vaccine strain. Compared to the common bimodal vaccine approach in which CTL and antibody responses are induced by the combination of a live recombinant vector (or a plasmid DNA) and a subunit immunogen, this recombinant *Salmonella* strain provides a relatively easier approach of single recombinant microbial vaccination. In addition, this vaccine strain can immunize via mucosal surfaces without the use of syringe injection, which would make this vaccination more acceptable from the patient's perspective. Thus, it would provide a new approach to the prevention of HIV/AIDS.

Acknowledgements

This work was supported by research grants from the Major State Basic Research Development Program of China (Nos. 2005CB522901 and 2006CB504200), the National Natural Science Foundation of China (Nos. 30470087 and 30570070), the Program for Changjiang Scholars and Innovative Research Team in University (No. IRT0745), the Department of Science Technology of Hubei Province (No. 2005ABC003), and the Science and Technology Programs of Wuhan (No. 200760323102) to J. Wu.

References

Amara, R.R., Robinson, H.L., 2002. A new generation of HIV vaccines. Trends Mol. Med. 8 (10), 489–495.

Breton, M., Zhao, C., Ouellette, M., Tremblay, M.J., Papadopoulou, B., 2007. A recombinant non-pathogenic Leishmania vaccine expressing human immunodeficiency virus 1 (HIV-1) Gag elicits cell-mediated immunity in mice and decreases HIV-1 replication in human tonsillar tissue following exposure to HIV-1 infection. J. Gen. Virol. 88 (1), 217–225.

Devico, A.L., Fouts, T.R., Shata, M.T., Kamin-Lewis, R., Lewis, G.K., Hone, D.M., 2002. Development of an oral prime-boost strategy to elicit broadly neutralizing antibodies against HIV-1. Vaccine 20 (15), 1968–1974.

Egan, M.A., Chong, S.Y., Rose, N.F., Megati, S., Lopez, K.J., Schadeck, E.B., Johnson, J.E., Masood, A., Piacente, P., Druilhet, R.E., Barras, P.W., Hasselschwert, D.L., Reilly, P., Mishkin, E.M., Montefiori, D.C., Lewis, M.G., Clarke, D.K., Hendry, R.M., Marx, P.A., Eldridge, J.H., Udem, S.A., Israel, Z.R., Rose, J.K., 2004. Immunogenicity of attenuated vesicular stomatitis virus vectors expressing HIV type 1 Env and SIV Gag proteins: comparison of intranasal and intramuscular vaccination routes. AIDS Res. Hum. Retroviruses 20 (9), 989–1004.

Fouts, T.R., Lewis, G.K., Hone, D.M., 1995. Construction and characterization of a *Salmonella typhi*-based human immunodeficiency virus type 1 vector vaccine. Vaccine 13 (6), 561–569.

Galen, J.E., Gomez-Duarte, O.G., Losonsky, G.A., Halpern, J.L., Lauderbaugh, C.S., Kaintuck, S., Reymann, M.K., Levine, M.M., 1997. A murine model of intranasal immunization to assess the immunogenicity of attenuated *Salmonella typhi* live vector vaccines in stimulating serum antibody responses to expressed foreign antigens. Vaccine 15 (6–7), 700–708.

Giri, M., Ugen, K.E., Weiner, D.B., 2004. DNA vaccines against human immunodeficiency virus type 1 in the past decade. Clin. Microbiol. Rev. 17 (2), 370–389.

Hone, D.M., Lewis, G.K., Beier, M., Harris, A., McDaniels, T., Fouts, T.R., 1994. Expression of human immunodeficiency virus antigens in an attenuated *Salmonella typhi* vector vaccine. Dev. Biol. Stand. 82, 159–162.

Kawahara, M., Hashimoto, A., Toida, I., Honda, M., 2002. Oral recombinant *Mycobacterium bovis* bacillus Calmette-Guérin expressing HIV-1 antigens as a freeze-dried vaccine induces long-term HIV-specific mucosal and systemic immunity. Clin. Immunol. 105 (3), 326–331.

Kotton, C.N., Hohmann, E.L., 2004. Enteric pathogens as vaccine vectors for foreign antigen delivery. Infect. Immun. 72 (10), 5535–5547.

Kotton, C.N., Lankowski, A.J., Scott, N., Sisul, D., Chen, L.M., Raschke, K., Borders, G., Boaz, M., Spentzou, A., Galán, J.E., Hohmann, E.L., 2006. Safety and immunogenicity of attenuated *Salmonella enterica serovar* Typhimurium delivering an HIV-1 Gag antigen via the Salmonella Type III secretion system. Vaccine 24 (37–39), 6216–6224.

Lorin, C., Mollet, L., Delebecque, F., Combredet, C., Hurtrel, B., Charneau, P., Brahic, M., Tangy, F., 2004. A single injection of recombinant measles virus vaccines expressing human immunodeficiency virus (HIV) type 1 clade B envelope glycoproteins induces neutralizing antibodies and cellular immune responses to HIV. J. Virol. 78 (1), 146–157.

Luo, F., Feng, Y., Liu, M., Li, P., Pan, Q., Jeza, V.T., Xia, B., Wu, J.G., Zhang, X.L., 2007. Type IVB pilus operon promoter controlling expression of the severe acute respiratory syndrome-associated coronavirus nucleocapsid gene in *Salmonella enterica serovar* Typhi elicits full immune response by intranasal vaccination. Clin. Vaccine Immunol. 14 (8), 990–997.

Ogra, P.L., Faden, H., Welliver, R.C., 2001. Vaccination strategies for mucosal immune responses. Clin. Microbiol. Rev. 14 (2), 430–445.

Ondondo, B.O., Yang, H., Dong, T., di Gleria, K., Suttill, A., Conlon, C., Brown, D., Williams, P., Rowland-Jones, S.L., Hanke, T., McMichael, A.J., Dorrell, L., 2006. Immunisation with recombinant modified vaccinia virus Ankara expressing HIV-1 gag in HIV-1-infected subjects stimulates broad functional CD4+ T cell responses. Eur. J. Immunol. 36 (10), 2585–2594.

Perri, S., Greer, C.E., Thudium, K., Doe, B., Legg, H., Liu, H., Romero, R.E., Tang, Z., Bin, Q., Dubensky, T.W., Vajdy, M., Otten, G.R., Polo, J.M., 2003. An alphavirus replicon particle chimera derived from venezuelan equine encephalitis and sindbis viruses is a potent gene-based vaccine delivery vector. J. Virol. 77 (19), 10394–10403.

Pickett, T.E., Pasetti, M.F., Galen, J.E., Sztein, M.B., Levine, M.M., 2000. In vivo characterization of the murine intranasal model for assessing the immunogenicity of attenuated *Salmonella enterica serovar* Typhi strains as live mucosal vaccines and as live vectors. Infect. Immun. 68 (1), 205–213.

Rayevskaya, M., Kushnir, N., Frankel, F.R., 2003. Anti-human immunodeficiency virus-gag CD8+ memory T cells generated in vitro from Listeria-immunized mice. Immunology 109 (3), 450–460.

Sacha, J.B., Chung, C., Rakasz, E.G., Spencer, S.P., Jonas, A.K., Bean, A.T., Lee, W., Burwitz, B.J., Stephany, J.J., Loffredo, J.T., Allison, D.B., Adnan, S., Hoji, A., Wilson, N.A., Friedrich, T.C., Lifson, J.D., Yang, O.O., Watkins, D.I., 2007. Gag-specific CD8+ T lymphocytes recognize infected cells before AIDS-virus integration and viral protein expression. J. Immunol. 178 (5), 2746–2754.

Stephens, J.C., Darsley, M.J., Turner, A.K., 2006. Stabilization of a plasmid coding for a heterologous antigen in *Salmonella enterica* serotype typhi vaccine strain CVD908-htrA by using site-specific recombination. Infect. Immun. 74 (7), 4383–4386.

Tan, G.S., McKenna, P.M., Koser, M.L., McLinden, R., Kim, J.H., McGettigan, J.P., Schnell, M.J., 2005. Strong cellular and humoral anti-HIV Env immune responses induced by a heterologous rhabdoviral prime-boost approach. Virology 331 (1), 82–93.

Tatalick, L.M., Gerard, C.J., Takeya, R., Price, D.N., Thorne, B.A., Wyatt, L.M., Anklesaria, P., 2005. Safety characterization of HeLa-based cell substrates used in the manufacture of a recombinant adeno-associated virus-HIV vaccine. Vaccine 23 (20), 2628–2638.

Vinner, L., Therrien, D., Wee, E., Laursen, I., Hanke, T., Corbet, S.L., Fomsgaard, A., 2006. Immune response in rhesus macaques after mixed modality immunisations with DNA, recombinant adenovirus and recombinant gp120 from human immunodeficiency virus type 1. APMIS 114 (10), 690–699.

Williams, J.G., Szalay, A.A., 1983. Stable integration of foreign DNA into the chromosome of the cyanobacterium Synechococcus R2. Gene 24 (1), 37–51.

Wolitski, R., Humfleet, G., Lee, J., Corby, N., 1992. HIV risk-related practices of male homosexual, bisexual, and heterosexual injection drug users. Int. Conf. AIDS 19–24 (8), C289.

Wu, H.Y., Nguyen, H.H., Russell, M.W., 1997. Nasal lymphoid tissue (NALT) as a mucosal immune inductive site. Scand. J. Immunol. 46 (5), 506–513.

Xu, F., Hong, M., Ulmer, J.B., 2003. Immunogenicity of an HIV-1 gag DNA vaccine carried by attenuated Shigella. Vaccine 21 (January (7–8)), 644–648.