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A type IVB pili operon promoter controlling nucleocapsid gene expression of SARS-CoV in *Salmonella* elicits full immune response by intranasal vaccination

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ABSTRACT

Attenuated *Salmonella enterica serovar typhi* have been implicated attractive as potential live oral delivery vector vaccines because of their ability to elicit the full array of immune responses in humans. In this study, we constructed an attenuated *S. enterica serovar typhi* strain stably expressing conserved nucleocapsid (N) protein of SARS-CoV by integrating the *N* gene into the *pilV* gene which was under the control of the type IVB pili operon promoter in *S. enterica serovar typhi*. BALB/c mice were immunized with this recombinant strain through different immune routes, viz intranasaly (i. n.), orogastricaly (o. g.), intraperitonealy (i. p.), and intravenously (i. v.). Results showed that i. n. route caused the highest production of specific IgG, IgG2a, and SIgA, where IgG2a was imprinted as a Th1 cell bias. Moreover, this recombinant live vaccine induced significantly high levels of specific cytotoxic T lymphocytes (CTL) activities, and increased IFN-gamma producing T cells compared with the parental strain. Our work provide insights that the type IVB pili operon promoter controlling SARS-CoV *N* gene expression in *Salmonella* might be an attractive live vector vaccine against the infection of SRAS-CoV for it could induce mucosal, humoral, and cellular immune responses. Our work also indicates that the type IVB pili operon promoter controlling foreign gene expression in *Salmonella* can elicit full immune responses by intranasal vaccination.

Key words: *Salmonella* delivery vector; SARS-CoV vaccine; humoral immune response; CTL activities; mucosal immunity

INTRODUCTION

Severe acute respiratory syndrome associated coronavirus (SARS-CoV), a type of coronavirus, is a new emerging virus and causes a high mortality rate along with a huge economic impact worldwide. It contains five major open reading frames encoding the replicase polyprotein, the Spike (S), the envelope (E), the membrane glycoprotein (M), and the nucleocapsid protein (N). N protein of SARS-CoV is a 46-kDa conserved protein that participates in the replication and transcription of the virus and interferes with the cell cycle of host cells (36). Amino acid sequence homology between SARS-CoV and other coronaviruses is low, and this is a possible cause for the difference in pathogenesis (25). It was reported that more than 94% of SARS patients were positive for N-protein specific antibodies that appeared at early stages of infection (41).

Other studies showed that the highest immune responses were generated by DNA vaccination with N gene against SARS-CoV in mice among the constructs encoding N, M, and E proteins (18). Therefore, the N protein was chosen as the target antigen in this study.

Currently no vaccine is licensed for human SARS-CoV, although effective vaccines have been developed for other animal coronaviruses (33). Recently, several vaccine strategies have been examined for prevention against SARS infection. They include inactivated viruses, DNA vaccine, and recombinant viral vectors based on adenoviruses, vaccinia viruses, and parainfluenza viruses (2, 3, 14, 35). In addition, the surface-displayed SARS-CoV spike protein on Lactobacillus casei has been reported to induce neutralizing antibodies in mice (22, 23). Studies of animal coronavirus vaccines

have demonstrated that both systemic and cell-mediated immune responses are important in preventing the viral infections (17). DNA vaccines targeting the nucleocapsid (N) protein of SARS-CoV generated strong N-specific humoral and cellular immunity and reduced the titers of challenging vaccinia virus expressing the N protein of the SARS virus (20). SARS-CoV N protein and S protein could induce a long persistence of memory T-cell response in humans (31, 42, 43). These evidences implicated that both humoral and cellular immune responses were vital to defense against acute SARS infection (34).

The initial SARS-CoV infection occurs primarily in the mucosal epithelial cells in the respiratory tract. There is evidence that mucosal immunity is important in the rapid immune response to mucosal infections (43). Thus, in order to develop an effective vaccine for SARS-CoV, a good SARS vaccine candidate should elicit both mucosal and systemic immunities.

Attenuated *Salmonella* strains have been supposed as attractive potential live oral delivery vector vaccines because of their ability to elicit a full array of immune responses in humans (1, 8, 10, 21, 24, 27). Previously we demonstrated that *S. enterica serovar typhi* mutant strain *pilS*::Km^R significantly attenuated the adhesion/invasion to human intestinal or monocytic cells compared with those of the wild type strain (28). The type IVB *pil* operon in *S.* Typhi contains 11 genes, *pilLMNOPQRSTUV*, of which *pilS* encodes the main structural protein and *pilV* codes for pilus-tip adhesion of the type IVB pili. These genes form a transcriptional unit under the control of one *pil* promoter, as evidenced by the absence of independent promoter. In this study, we sought to obtain an effective *Salmonella*-based viral vaccine candidate with both mucosal and systemic

immunities. We constructed a *S. enterica serovar typhi* mutant strain *pilS*::Km^R *pilV*:: N^+ by integrating the *N* gene to the *pilV* gene under the control of *pil* operon promoter as the first step towards constructing a live oral *Salmonella*-SARS-CoV vaccine and analyzed its specific immune responses in mice with different immune routes.

MATERIALS AND METHODS

Strains and plasmids

The attenuated *S. enterica serovar typhi* Ty2 (*cys try galE*-H1 *via rif*^{R} *pilS kan*^{R}) (15, 44), was the source for the vaccine strain in this study. The eukaryotic expression vector pCMV-Tag2B-N expressing full length nucleocapsid (N) gene of SARS-CoV strain (WHU) was as described previously (46).

Construction of the recombinant *S. enterica serovar typhi* *pilS*::Km^{R} *pilV*::N^{+} vaccine stably expressing N protein of SARS-CoV

The most important approach used in the construction of recombinant vaccine is homologous recombination. Plasmid pUST100 containing the *Apa*I-*Eco*RV digestive sites fragment with *pilV* and *rci* genes (45) was cleaved with *Bam*HI, which generated a small and a large fragment where then the large fragment was religated to generate a new plasmid named pUST110. The *N* gene (1.2-kb) of SARS-CoV was obtained by digestion of pCMV-Tag2B-N with *Bam*HI and *Eco*RI and then inserted into the vector pUST110 digested with *Bgl*II and *Eco*RI to create a recombinant plasmid pUST110-N. Plasmid pUST110-N was transferred into *S. enterica serovar typhi pilS*::Km^{R} via the *S. enterica serovar typhimurium* modifying strain J357 ($r^{-}m^{+}$) (44) with Ap^{R} and Km^{R} selection. Individual clones were then grown in 5 ml LB without antibiotic selection at 42^{o}C for periods of *ca*. 24 h before transfer of a 0.1 ml aliquot into 5 ml fresh medium. After 3-4 transfers, aliquots were plated onto LB containing kanamycin and resulting colonies that had lost the transformed plasmids were checked for ampicillin sensitivity. The

recombinant *S. enterica serovar typhi pilS*::Km^R *pilV*::N^+ vaccine, in which the *N* gene was inserted into the *S. enterica serovar typhi* genome, was screened from large numbers of colonies and examined by PCR amplification using primers adjacent to or inside the inserted fragment as well as by Western blot analysis.

PCR reaction was used to identify the *N* gene in *S. enterica serovar typhi pilS*::Km^R *pilV*::N^+ strain. Primers outside the recombinant sequences used for amplification were as follows: 5'-CGATGATAGTCCGGAATCAGC-3', and 5'-ATCCGGACGACCATTGACCTG-3'. Primers with insert sequences used for amplification were as follows: 5'-ATGTCTGATAATGGACC-3', and 5'-TGCCTGAGTTGAATCAG -3'. The expression of N protein in *S. enterica serovar typhi pilS*::Km^R *pilV*::N^+ was determined by Western blot analysis.

Immunization process optimization

Seven to eight weeks old female BALB/C mice were prepared for immunization. Four groups (six mice per group) of mice were immunized with the recombinant strain or the parental bacterium strain through: (a) i. n. by dispensing 10 μl of vaccine suspension containing 10^9 CFU, directly into the mouse nasal cavity; (b) o. g. by placing 100 μl of vaccine suspension containing 10^9 CFU into the lower esophagus using a gavage needle; (c) i. p. and (d) i. v. by injecting 100 μl of vaccine suspension containing 10^7 CFU into abdominal cavity and vena caudalis respectively on day 0 and 14. Mice were euthanized at day 14 after the last immunization. Production of serum IgG against N protein was measured by enzyme-linked immunosorbent assay (ELISA).

Purification of recombinant N protein and antibody production

The *N* gene of SARS-CoV was generated by digestion of pCMV-Tag2B-N (46) with *Bam*HI and *Eco*RI and cloned into the *Bam*HI and *Eco*RI sites of prokaryotic vector pGEX-KG to yield plasmid pGEX-N. Plasmid pGEX-N was transformed into *E. coli* BL21(DE3)[pLysS]. The GST-N fusion protein was overexpressed in *E. coli* after the induction of IPTG. The expressed protein was purified by Glutathione sepharose 4B (Amersham Biosciences). Anti-N antibody was prepared from immunized rabbit sera with the recombinant GST-N protein.

Measurement of antibody levels by ELISA

Anti-N antibodies were detected two weeks after the final immunization. The levels of antibodies against N protein were determined by ELISA, respectively. Briefly, 96-well plates were coated with 100 μl of the recombinant N protein (10 μg/ml) (expressed and purified from *E. coli* BL21) in carbonate buffer, pH9.6, for 3h at 37°C and blocked overnight with 1% bovine serum albumin at 4°C. After incubation, the plates were washed with PBS containing 0.05% Tween-20 (PBST). Sera were subjected for titer determination in twofold serial dilutions. Antibodies bound to the immobilized antigens were detected using horseradish peroxidase-labeled anti-mouse IgG, IgG2a (Southern Biotech) diluted at a ratio of 1:1,500 in PBST and substrate solution contained O-phenylenediamine (1 mg/ml) and H_2O_2 (0.03%) in 0.1 M citrate-phosphate buffer. Test and control sera were run in triplicate.

Detection of anti-N SIgA antibody by ELISA

Briefly, flat-bottomed microtiter plates were coated with the recombinant N protein (expressed and purified from *E. coli* BL21) and blocked with 1% bovine serum albumin. The supernatant extracted from 100 μg original fecal pellets of each mouse mixed with 100 μl of phosphate-buffered saline was subjected to titer determination in twofold serial dilutions. Samples were incubated at 100μl/well for 1h at 37°C. Horseradish peroxidase-conjugated goat anti-mouse IgA antibodies (Southern Biotech) were added at a 1:5,000 dilution in PBST and incubated at 37°C for 1h. Experimental and control samples were run in triplicate.

Stable transfection

Murine colon tumor cells CT26 (H-2^d) (1 x 10^6) cells were placed into 60-mm-diameter plate 24 h before transfection. Each of 60-mm-diameter plate of cells was transfected with 8 μg plasmid pCMV-Tag2B-N by lipofectamine^{TM} 2000 (Invitrogen). For stable transfections, G418 was added to the cell culture media at a final concentration of 0.6 mg/ml after 48 h post-transfection. Cell culture medium was changed every 2 days. After 4 weeks of selection, N-expressing CT26 cells were obtained. Cells from wells containing single clones were selected for further analysis and individual transfected clones were tested for expression of N protein by Western Blot analysis.

Specific cytotoxic T lymphocytes (CTL) activity

Seven to eight weeks old female BALB/C mice were immunized with 10^9 CFU/10μl

of the recombinant strain or the parental bacterium strain intranasaly on day 0 and 14, respectively. Mice were euthanized at day 28. Splenocytes from mice were resuspended in complete RPMI-1640 with 10% FBS and analyzed for CTL activity. Twofold serial dilutions of mice splenocytes as expanded effector cells (6.25×10^3-2×10^5cells/wells) were stimulated by recombinant N protein (5 μg/ml, expressed and purified from *E. coli* BL21) *in vitro* and incubated with MHC-matched CT26 (H-2^d), which stably expressed N protein as target cells (2×10^4 cells/wells) for 4 h at 37°C in the presence of 5% CO_2. Cultures were centrifuged at 1000×rpm for 5 min and 50 μl supernatants per well were then transferred to enzymatic assay plates, and lysis was determined by measuring released lactate dehydrogenase (LDH) by using the Cytotoxic 96 assay kit (Promega Corp., Madison, Wis.). The absorbance values from the supernatants were recorded at 490 nm on an ELISA microplate reader. The data are means ±SD of five different wells. The percentage of cytotoxicity was calculated as follows: (experimental – effecter spontaneous – target spontaneous)/(target maximum – target spontaneous) × 100, where spontaneous release is the count released by target cells in the absence of effecter cells and maximal release is the counts released in the presence of lysis solution.

ELISPOT assay

Ninety six well filtration plates were coated overnight at 4°C with 50 μl (10 μg/ml) of anti-mouse IFN-γ or IL-4 in sterile PBS. The plates were blocked for 2 h at 37°C with RPMI-1640 containing 10 % FCS and 1 % BSA and were washed three times with sterile PBS. Various dilutions of splenocytes from immunized or control mice in 200 μl of complete medium were placed in each well and were cultured for 18 h in RPMI-1640

alone (negative control) or with 5 μg per ml recombinant N protein (expressed and purified from *E. coli* BL21) or 8 μg per ml of concanavalin A (positive control) in triplicate wells incubated at 37°C for 24 h. Plates were washed with PBS containing 0.025 % Tween-20 and were overlaid with 50 μl (5 μg/ml) of biotinylated anti-mouse IFN-γ or IL-4. The plates were washed six times with PBS containing 0.025% Tween-20 and were treated with 1.25 μg of avidin-conjugated alkaline phosphatase (Sigma) per ml for 2 h at room temperature. After a final wash with PBS, IFN-γ or IL-4 spot-forming cells were detected by the addition of BCIP-nitroblue tetrazolium solution (Sigma) and were counted with a stereomicroscope.

***In vivo* expansion of CTL effectors in tumor protection model**

Seven to eight weeks old female BALB/C mice were immunized intranasaly with 10 μl of vaccine suspension containing 10^9 CFU on day 0 and 14 as primary and booster immunizations. At day 14 after the last immunization, mice were given subcutaneous injections into the right flank of CT26 target cells which stably expressing N protein. The tumor sizes were measured using calipers every three days. Tumor volumes were calculated according to the formula: volume= $\text{width}^2 \times \text{length} \times 0.52$. The data are presented as means±S.E.

Statistical analysis

One-way ANOVA and Student's t tests were used for comparison of antibody titers, cytotoxicity levels, tumor growth and IFN-γ or IL-4 production by ELISPOT among

different groups. All tests were performed using SPSS software. Values of $p<0.05$ are considered significant.

RESULTS

Construction of recombinant *S. enterica serovar typhi* *pilS*::Km^R *pilV*::N^+ strain.

The recombinant *S. enterica serovar typhi pilS*::Km^R *pilV*::N^+ strain carrying *N* gene of SARS-CoV was constructed according to the procedures described in the materials and methods section and shown in figure 1A. The vaccine strain was confirmed by PCR amplification using the primers outside recombinant sequences (in which *N* gene was inserted into the *pilV* gene) (Fig. 1B). The size of PCR product was 1500 bp from the recombinant *S. enterica serovar typhi pilS*::Km^R *pilV*::N^+ vaccine strain (Fig. 1B), while the PCR product from the parental strain *S. enterica serovar typhi pil*S::Km^R was 300 bp (Fig. 1B). PCR using the primers with insert sequences (Fig. 1C) also indicated that the *N* gene (1200 bp) was inserted into the *pilV* gene of the genomic DNA in the recombinant *S. enterica serovar typhi pilS*::Km^R *pilV*::N^+ strain.

We further examined the N protein expression in the recombinant *S. enterica serovar typhi pilS*::Km^R *pilV*::N^+ strain with SDS-PAGE and Western blot analysis. As shown in figure 1D, a protein band of approximately 45-kDa (corresponding to the molecular mass of N protein) in the cell lysates of the *S. enterica serovar typhi pilS*::Km^R *pilV*::N^+ strain (Fig. 1D, lane 2) was identified, but not detected in the cell lysates of the parental bacterium strain *S. enterica serovar typhi pil*S::Km^R (Fig. 1D, lane 1). The results demonstrated that the N protein of SARS-CoV was expressed in the newly constructed *S. enterica serovar typhi pilS*::Km^R *pilV*::N^+ recombinant strain. We have demonstrated the stability of insertion and expression of vaccine strain after several *in vitro* passages.

S. enterica serovar typhi pilS::Km^R_pilV_::N$^+$ vaccine strain elicited predominant IgG2a subclass antibody responses.

Sera IgG responses against N protein were determined in the BALB/C mice immunized with the recombinant *S. enterica serovar typhi pilS*::Km^R *pilV*::N$^+$ vaccine strain through different immune routes, including i. n., o. g., i. p., and i. v. Results from ELISA analysis showed that i. n. immune route caused the highest levels of anti-N protein specific IgG and IgG2a (Figs. 2A and 2B) (i. n. VS o. g., i. p., and i. v., $*p< 0.05$). In addition, both i. n. and o. g. immune routes induced the highest levels of specific SIgA among all immune routes (Fig. 2C). The parental strain (with no insert) and non immunized mice (controls) did not induce a detectable antibody response. The data suggested that i. n. immunization was the best route for this recombinant vaccine. Therefore, we chose i. n. immunizing route for the rest of the experiments in this study. In mice, the presence of IgG2a antibodies is indicative of a Th1-biased response since the Th1 cytokines are necessary for this isotype shift in B cells. Our results demonstrated that immunization of mice with the recombinant *S. enterica serovar typhi pilS*::Km^R *pilV*::N$^+$ strain could induce more of an IgG2a Th1-type response than the parental strain. In addition, the recombinant strain could elicit specific mucosal immunity.

Recombinant _S. enterica serovar typhi pilS_::Km^R _pilV_::N$^+$ vaccine stimulated specific CTL responses in mice.

Mice were immunized with the recombinant strain or the parental bacterium strain intranasaly on day 0 and 14, respectively, and then euthanized at day 28. The specific

CTL activities in splenocytes from the immunized mice were examined. Splenocytes from immunized mice were restimulated with the recombinant N protein *in vitro* as expanded effector cells, and incubated with MHC-matched CT26 (H-2^d), which stably expressed N protein as target cells. CTL assays showed that the recombinant vaccine strain elicited significantly higher levels of specific CTL responses than the parental strain *S. enterica serovar typhi pil*S::Km^R and the control group (Fig. 3). We determined that the highest CTL activity was detected when the ratio of effecter cell/target cell (E/T) was 2.5:1. These results demonstrated that the recombinant *S. enterica serovar typhi pilS*::Km^R *pilV*::N^+ vaccine strain could significantly stimulate specific CTL responses in mice.

Recombinant *S. enterica serovar typhi pilS*::Km^R *pilV*::N^+ vaccine induced IFN-γ production.

IFN-γ secretion is indicative of a Th1-biased response, while IL-4 production is indicative of a Th2-biased response. The production of IFN-γ and IL-4 were detected from splenocytes in all immunized mice groups using the ELISPOT technique. After stimulation by the recombinant N protein (expressed and purified from *E. coli* BL21), splenocytes from the recombinant strain group induced significantly higher levels of IFN-γ producing cells (mean± SD, 5270± 244) than those from the parental strain (mean± SD, 2300± 268) group (*p<0.05) (Fig. 4A). However, there were no significant differences of the expressions of IL-4 producing cells among all groups including the recombinant vaccine group, parental strain group, and non-immunized control group (Fig. 4B). This indicated that the recombinant strain induced a Th1-biased immune response.

Recombinant *S. enterica serovar typhi pilS*::Km^{R} *pilV*::N^{+} vaccine inhibited the N-expressing tumors growing *in vivo*.

Specific cellular immune response is a crucial mechanism of defense against intracellular microorganisms, viruses and tumors. In this study, BALB/c mice were immunized with the recombinant vaccine or the parental strain. At day 14 after the last immunization, mice were given subcutaneous injections of CT26 target cells which stably expressing N protein. The CD8+T cells of BALB/c mice recognized MHC-I matched CT26 cells and protected BALB/c mice from CT26 tumor challenge. The results showed that the tumor sizes in the recombinant vaccine group were significantly smaller than those in the control parental strain group (* $p<0.05$) (Figs. 5A and 5B). The sizes of tumors increased singificantly in a time dependent fashion in the parental strain group, but insignificantly in the recombinant vaccine group (Fig. 5A). These data showed that the recombinant strain could inhibit the N-expressing tumors growing *in vivo* by increasing specific cellular immune response.

DISCUSSION

S. enterica serovar typhi are attractive for their use as vaccine vectors since they can be administered by the natural route of infection, i.e. orally, and are capable of eliciting both systemic and mucosal immune responses (11, 15, 37, 38). Of the more than 2300 closely related *S.* serovar recognized, *S. enterica serovar typhi* is the only one that invades the bloodstream and causes systemic infection and immunity exclusively to humans. Some attenuated *S. typhi* strains (for example *S.* Typhi Ty21a) have been reported to be used for expression of heterologous antigens/proteins that can be delivered to the immune system in human studies (21). The possibility of using *S. typhi* as a live vector to express heterologous antigens or deliver them in genetic form in a multivalent vaccine that could protect against several diverse pathogens is an attractive alternative in vaccine development and delivery (4, 5, 6, 7, 16, 24).

In this study, the reason we selected *S. enterica serovar typhi pil*S::Km^R strain as the parental bacterium is that the expressions of virulent antigen Vi and type IVB pili are defective (44). The type IVB pilus of the pathogen has been identified as an important virulence factor that was required for pathogenicity (44). Moreover, the type IVB pilus operon is confined to *S.* Typhi and a few other human–invasive strains such as *Salmonella enterica* serovar Paratyphi C and Dublin (26, 40). *S. enterica serovar* typhi mutant strain (*pilS*::Km^R) significantly attenuated the adhesion/invasion to human intestinal or monocytic cells compared with those of the wild type strain (44, 28). Of the attenuated *S. typhi* strains (for example *S.* Typhi Ty21a), the immunogenicity of Ty21a is not remarkable, particularly when the vaccine is administered in capsule form by oral

vaccination. Other highly immunogenic *S. typhi* dervatives made and tested to date have either caused fever, or may retain the potential to cause fever, in at least some small numbers of volunteer recipients. So the attenuated *S. enterica serovar typhi pil*S::Km^R strain was examined in this study for its possibility to elicit full and strong immune response by the natural route of infection. Our results clearly showed that the type IVB pili operon promoter controlling nucleocapsid gene expression of SARS-CoV in *S. enterica serovar typhi pilS*::Km^R*pilV*::N^+ could elicit full specific immune response by intranasal vaccination.

Since plasmid DNA is relatively easily lost, we constructed this attenuated *S. enterica serovar typhi* vaccine stably expressing the conserved N protein of SARS-CoV by integrating the *N* gene into the *pilV* gene under the control of *pil* promoter in the chromosome of the strain. This expression of N protein in vaccine strain is stable after many (more than 20) *in vitro* passages (data not shown). However, this attenuated *S. enterica serovar typhi* strain is a Ty2 derivative, EX462, with rifampin-resistant, Vi-negative, gal mutant, and with a kanamycin cassette inserted to inactive *pilS* (44). Therefore, this strain encodes resistance to two antibiotics, which limits the animal experimental and clinical studies. For this reason, future work may be performed to insert other candidate antigen genes (for example, SARS viral Spike) into the two antibiotic genes (Rif^R, Kam^R) to confer their resistance.

In recent years, the intranasal dosing model has been used to demonstrate the immunogenicity of *S.* Typhi vaccine strains in a number of other studies (9, 12, 29, 30，32). For example, *S. typhi* CVD 908 expressing the major protein gp63 of *Leishmania mexicana mexicana* was shown to be immunogenic through this pathway, eliciting both

humoral and cellular immune responses (12). Pickett et al.（32）reported that attenuated *S.* Typhi live vector vaccine strains were highly immunogenic in mice following intranasal but not orogastric inoculation. Despite the established utility of this murine intranasal model (1, 9), little is known about the immunogenicity of serovar Typhi vaccine strains administered by different routes. We demonstrated that the i. n. route of immunization caused the highest production of specific IgG, IgG2a and SIgA to SARS-CoV N antigens delivered by *S. enterica serovar typhi* live-vector vaccines compared to the routes of o. g., i. p., and i. v. (Fig. 2). In addition, 10^9 CFU of bacteria with i. v. or i. p. routes could cause death of mice (data not shown), while 10^7 CFU of bacteria with i. v. or i. p. route did not induce enough immunogenic responses (Fig. 2), so we considered that i. n. route was the best, although, the dose of i. n. and o. g. application was 100-fold higher than that for i. p. and i. v. application routes, which might have some effects on magnitude of response. More safety concerns about the i. n. route for live bacterial vector strains in humans might be considered for further investigation.

Cell-mediated immune response is a crucial mechanism of defense against most intracellular pathogens and tumors (13, 19), although it remains elusive for the new emerging SARS-CoV. Our data showed that the recombinant strain could inhibit the N-expressing tumor growing *in vivo* (Fig. 5) by stimulating specific cellular immune responses. We also observed that the attenuated *S. enterica serovar typhi* vaccine *pilS*::Km^R *pilV*::N^+ elicited strong specific CTL responses (Fig. 3) as measured by LDH release assay at ratio of E/T as 2.5/1. There was no significant increase of CTL responses at the E/T ratio of 5/1 and 10/1 (data not shown), and the reasons for these are not known. We speculate that one of the reasons might be due to minimal activity detected following

in vitro expansion of memory cells. Another possibility is that CTL activities are not measured by direct MHC class I perforin mediated lysis experiments.

There are several advantages for the recombinant attenuated *S. enterica serovar typhi pilS*::Km^R *pilV*::N^+ vaccine: firstly, it can elicit systemic immunity, and it is attenuated (28, 44) and relatively safe; secondly it can be used as a mucosal vaccine vector to deliver pathogen-specific protective epitopes into the mucosal-associated lymphoid tissues; thirdly, it is easy for manipulation, grows fast, and it costs much lower than viral vector vaccines or DNA vector vaccines. Both mucosal and systemic immune responses against the carrier and the foreign antigens may be obtained via i. n. route (29).

Until now, effective live vaccine, which can elicit both mucosal and systemic immune responses against SARS-CoV are very limited. Our new constructed attenuated *S. enterica serovar typhi pilS*::Km^R *pilV*::N^+ proved to be a highly proficient live vaccine candidate. Our works also provide insights that integration of other foreign genes into the genes under the control of type IVB pili promoter in the attenuated *S. enterica serovar typhi* live vector might be attractive in vaccine delivery and vaccine candidate development against intracellular microorganism infection, respiratory infection, or inhibiting tumor growth. This is due to its ability to stably express foreign antigens and induce mucosal, humoral, and cellular immune responses.

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FIGURE LEGENDS

Fig. 1. Construction and analysis of the recombinant *S. enterica serovar typhi pilS*::Km^R *pilV*::N$^+$ strain with chromosomal integration of *N* gene. (A). Diagram of the construction of recombinant strain. (B). Analysis of recombinant strain by PCR amplification using primers outside recombinant sequences. Lane 1: Parental strain *S. enterica serovar typhi pilS*::Km^R; Lane 2: Plasmid pUST110-N; Lane 3: Recombinant strain *S. enterica serovar typhi pilS::*Km^R *pilV::*N$^+$; Lane 4: DNA Marker. (C). Analysis of recombinant strain by PCR amplification using primers with insert sequences. Lane 1: DNA Marker; Lane 2: Recombinant strain *S. enterica serovar typhi pilS::*Km^R *pilV::*N$^+$; Lane 3: Parental strain *S. enterica serovar typhi pilS*::Km^R. (D). Examination of N protein (45 kD) expression in the recombinant strain *S. enterica serovar typhi pilS::*Km^R *pilV::*N$^+$ with anti-N antibody. Lane 1: Parental strain *S. enterica serovar typhi pilS*::Km^R; Lane 2: Recombinant strain *S. enterica serovar typhi pilS*::Km^R *pilV*::N$^+$.

Fig. 2. Determination of sera antibody responses against SARS-N protein. Each group of BALB/c mice (n=6) was immunized with the recombinant strain *S. enterica serovar typhi pilS*::Km^R *pilV*::N$^+$ through different immune routes, viz i. n., o. g., i. p., and i. v. The levels (titers) of sera IgG (A), IgG2a (B) and SIgA (C), produced in immunized mice were determined at day 14 after the last immunization with ELISA method. All of the experiments were repeated five times. The data are means ± S.E.

Fig. 3. Determination of N-specific CTL responses in mice immunized through i. n.

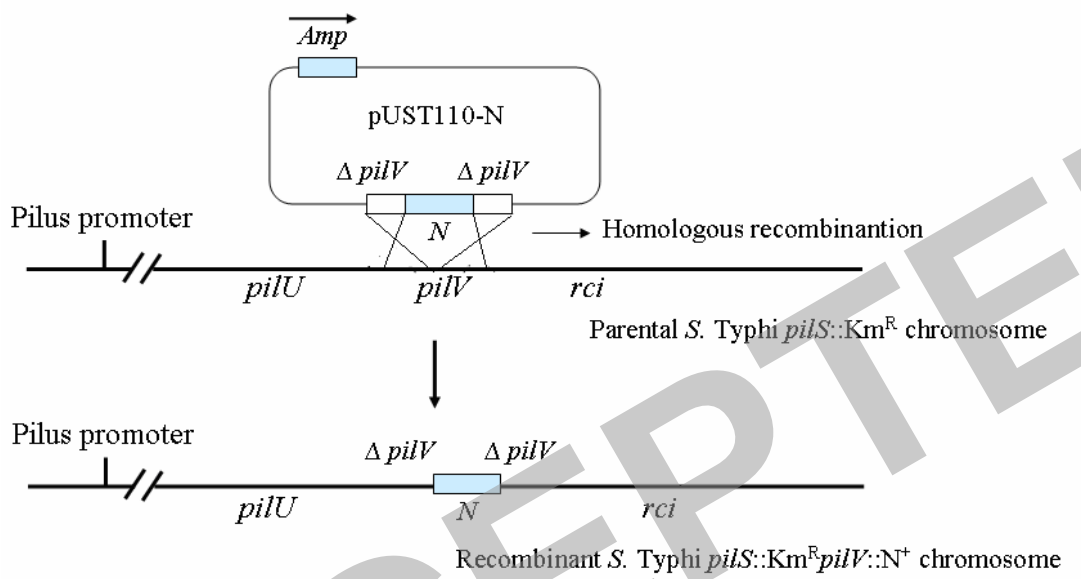
with recombinant vaccine strain. Each group of mice (n=6) was immunized with parental strain *S. enterica serovar typhi pilS*::Km^R or recombinant strain *S. enterica serovar typhi pilS*::Km^R *pilV*::N^+ intranasaly on day 0 and 14, respectively. Splenocytes were harvested at day 14 after the last immunization, and stimulated by the recombinant N protein as effector cells. MHC-matched CT26 ($H\text{-}2^d$) cells, which stably expressed N protein, were as target cells All columns showed the mean percentages of special cytotoxicity at E/T ratio of 2.5:1 in LDH release assay. The experiments were repeated six times. The data are means±S.E.

Fig. 4. Cytokines production. Each group of mice (n=6) was immunized with parental strain (*S. enterica serovar typhi pilS*::Km^R) or recombinant strain (*S. enterica serovar typhi pilS*::Km^R *pilV*::N^+). Productions of IFN-γ (A) and IL-4 (B) spot-forming-cells (SFC) per 10^6 splenocytes were assessed by ELISPOT assay using splenocytes isolated from immunized mice. All columns show the mean numbers of SFC, the error bars indicate standard deviations. A significant increase of IFN-γ secretion was observed in mice immunized with the recombinant *S. enterica serovar typhi pilS*::Km^R *pilV*::N^+ (*p<0.05).

Fig. 5. Analysis of specific anti-tumor activity of CTL responses in immunized mice. (A) The growth curve of tumor cells (*S. enterica serovar typhi pilS*::Km^R *pilV*::N^+ group VS parental strain *S. enterica serovar typhi pilS*::Km^R , * p< 0.05). The data are means± S.E. (B) Tumor sizes in immunized mice on the 20^{th} day post-injection with 2×10^6 CT26 tumor cells.

Figure 1

1A.



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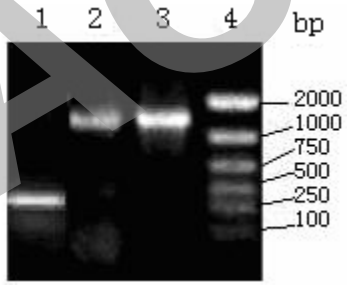

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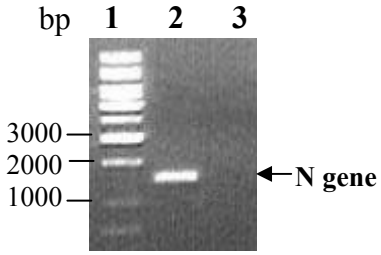

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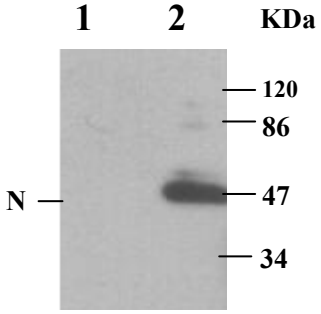
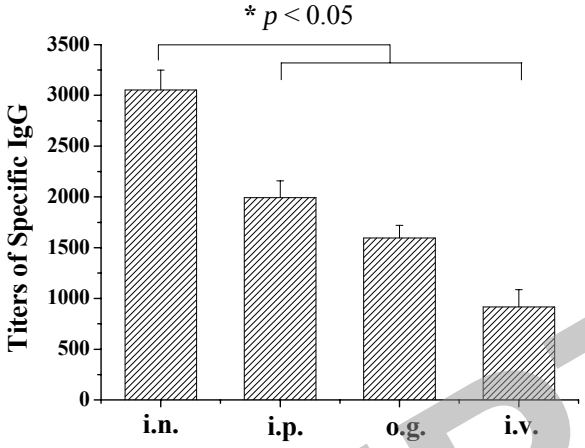


Figure 2

2A.



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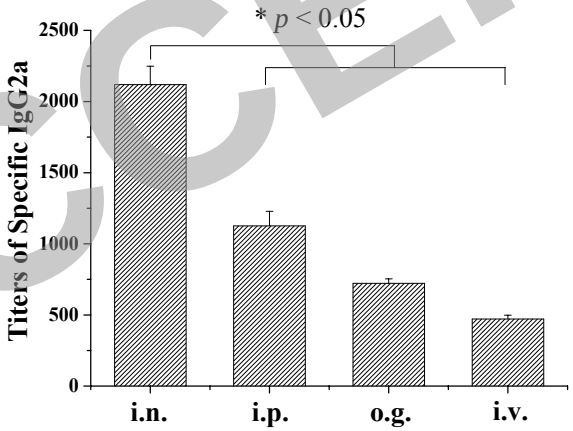

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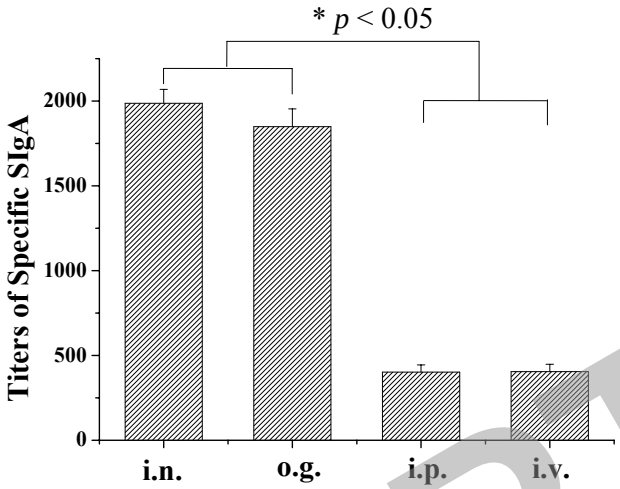


Figure 3

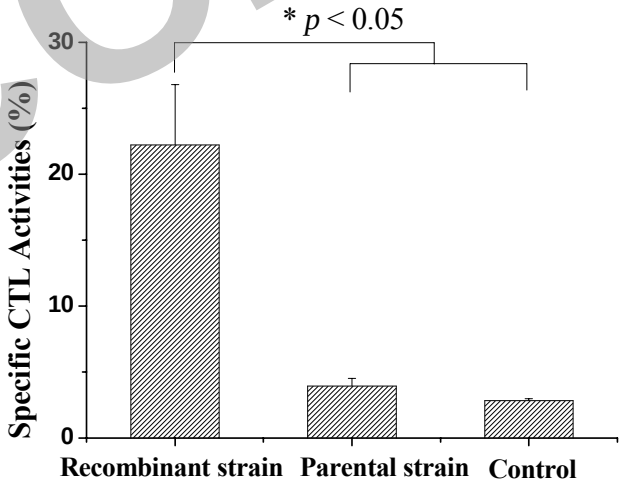
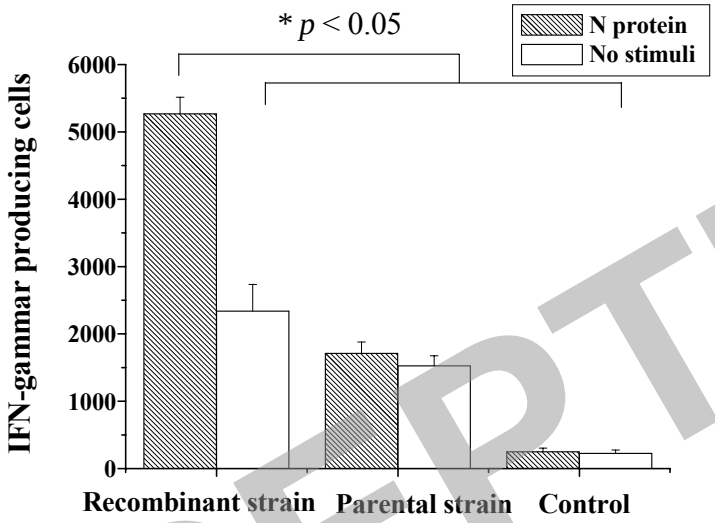


Figure 4

A



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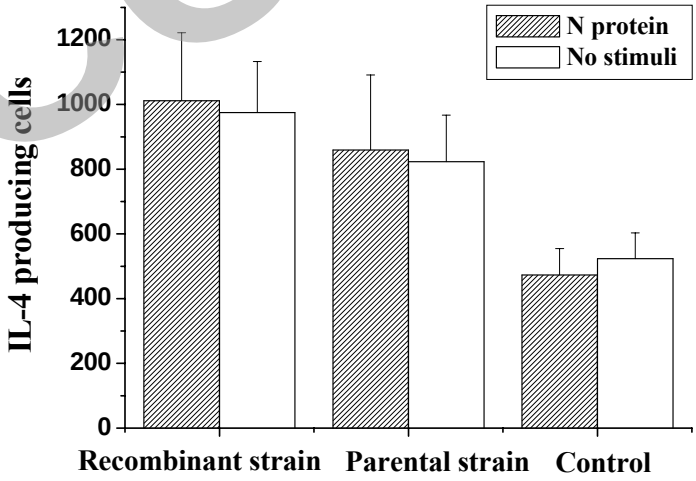
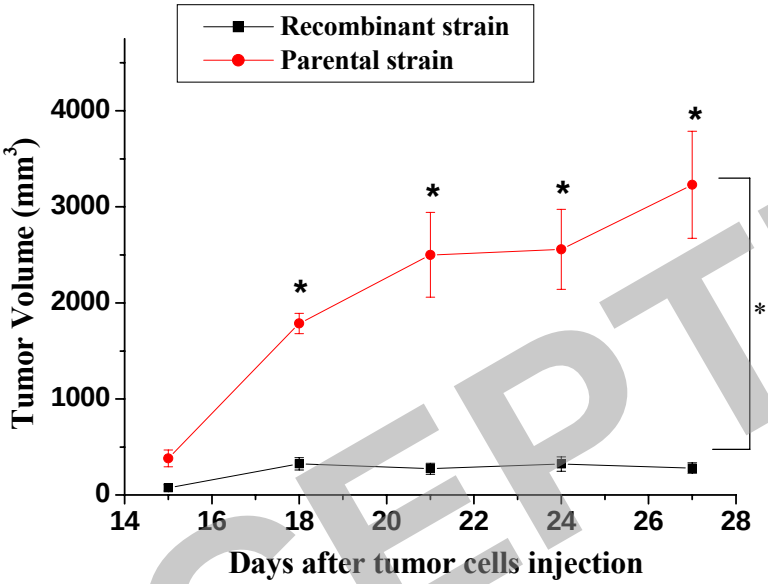


Figure 5

A



B

Day 20 after inoculation of tumor cells