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Two oral HBx vaccines delivered by live attenuated *Salmonella*: Both eliciting effective anti-tumor immunity

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Abstract

Live attenuated bacteria have great potential for use in vaccine development due to several unique advantages, including stable antigen expression, effective antigen presentation, convenient and inexpensive delivery, and low cost of vaccine production. In this study, we expressed hepatitis B virus x gene (HBx) on mouse melanoma cells as the target antigen and constructed *Salmonella*-based HBx vaccines by two strategies, i.e., recombinant eukaryotic plasmid encoding HBx and a recombinant prokaryotic plasmid encoding Type III secretion system effector-HBx fusion protein. Both HBx constructs elicited significant levels of CTL reaction and IFN-γ secreting T cells. When mice were challenged with melanoma cells expressing HBx, tumor growth rates in immunized animals were significantly slower than controls. Tumor sizes and tumor weight indices of immunized mice were also significantly lower than controls. We conclude that both strategies described in this study may lead to novel approaches of tumor vaccines.

Keywords: Tumor vaccine; *Salmonella*; Type III secretion system (TTSS); Hepatitis B virus x gene (HBx); Anti-tumor immunity

1. Introduction

Live attenuated bacteria have been used extensively in vaccine development due to their unique advantages as vaccine carriers: (1) stable *in vivo* antigen expression; (2) effective antigen presentation; (3) components of bacterial cells serving as effective immunological adjuvants; (4) convenient and inexpensive vaccine delivery with no associated trauma; and (5) low cost of vaccine production. Attenuated strains of *Salmonella* have all of these characteristics and have been well studied [1,2].

Salmonella can survive and multiply in macrophages and other antigen presenting cells (APCs) [3,4], which is a very useful feature of *Salmonella* as antigen carriers for vaccine development. There are reports indicating that live attenuated *Salmo-*

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nella bacterial cells in macrophages could release plasmids into eukaryotic cytoplasm by some unknown mechanism [5]. This finding has inspired the study and development of several vaccines with antigen sequence inserted in eukaryotic plasmids, which are then transformed into live attenuated *Salmonella* [5,6]. On the other hand, researchers are also exploring the bacterial type III secretion system (TTSS) for use in vaccine development. TTSS is a needle-like structure expressed on bacterial cell-surface [7,8]. It consists of two types of proteins: structural proteins and effectors. Effectors can be secreted and translocated into host cells via the TTSS needle complex. This translocation is mediated by the N- terminal amino acid sequence of effectors; changes in the C-terminal region of effectors do not interfere with translocation [9–11].

In *Salmonella*, there are two types of TTSSs, encoded by *Salmonella* Pathogenicity Island-1 (SPI-1) and *Salmonella* Pathogenicity Island-2 (SPI-2) and denoted as SPI-1 TTSS and SPI-2 TTSS, respectively [12–14]. The SPI-1 TTSS is expressed and assembled in the early stages of infection, during which host cell invasion by bacteria is facilitated by SPI-1 TTSS–host cell interaction [4,12]. In contrast, SPI-2 TTSS is mainly assembled in systemic infection stage, during which bacteria reside within phagocytic vacuoles of the host cells. SPI-2 TTSS ensures the necessary communication between bacteria and host cells [12]. Both SPI-1 TTSS and SPI-2 TTSS have their unique effectors, such as SopE, SopE2, and SptP for SPI-1, and SspH2 and SifA for SPI-2.

TTSS effectors, or effector-antigen fusion proteins, are secreted into the cytosol of macrophages so that they can be processed to initiate MHC-I restricted immune reactions [15–19]. In addition, these proteins may also start MHC-II restricted reactions via different mechanisms. Recently, Russmann and colleagues utilized SspH2-fusion proteins to immunize mice and achieved both significant CD8+ and CD4+ T cell responses [20]. In their studies, these researchers found that SspH2 works better than SopE2 or SifA in the fusion proteins in eliciting CD8+ or CD4+ T cell responses [20]. However, despite these exciting early results and some additional TTSS-based vaccine studies [15–19], no any such vaccine has been successfully developed to date.

Hepatocellular carcinoma (HCC) is one of the most prevalent forms of human cancer worldwide [21] and its development is strongly associated with infections with hepatitis viruses, including hepatitis B virus (HBV). HBx, an HBV-encoded protein with multiple functions in cellular signal transduction and HBV replication [22], has been recognized as an important oncogene, closely relevant with the carcinogenesis and development of HBV-induced HCCs [23–25]. Several cytotoxic T lymphocyte (CTL) epitopes have been mapped wiin HBx sequence [26,27]. Vaccines based on HBx full-length sequence or specific epitopes could elicit significant immune reactions [28]. However, immunogenicity of cancer antigens has been low, imposing a long-existing hurdle in anti-tumor immunology. Therefore, augmentation of the immunogenicity of cancer antigens has been a long sought goal in the development of effective vaccines. In this study, we attempted to take the advantages of *Salmonella* as a vaccine vector to elevate the immunogenicity of cancer antigens, using HBx as a representative tumor antigen in the experiments. We cloned HBx into *Salmonella* by two strategies, one into a eukaryotic plasmid and one into TTSS expressed as a protein fused to SspH2 (SspH2-HBx). When delivered orally to the animal, both vaccine strains elicited significant levels of protective immunity against the transplanted melanoma tumor that expressed the HBx antigen.

2. Materials and methods

2.1. Bacteria strains and cell lines

Salmonella typhimurium SL1344 and SL3261 were obtained from *Salmonella* Genetic Stock Center, SGSC (www.ucalgary.ca/~kesander). Bacterial strains were all cultured in LB broth or on LB-agar plates [29]. B16 C57BL/6J mouse melanoma cells (ATCC Nos. CRL-6475, H2-K^b) and RAW264.7 mouse macrophage cells (ATCC No. TIB-71) were generously provided by Dr. Hongyan Pang (Department of Pharmacology, Institute of Materia Medica, Chinese Academy of Medical Sciences & Peking Medical College, China). B16 cells were cultured in RPMI 1640 (Invitrogen) + 10% calf serum (Invitrogen), and RAW264.7 cells were cultured in DMEM (Invitrogen) + 10% calf serum.

2.2. HBx gene amplification, cloning, and transfection

HBV-carrying serum was collected from a female patient with acute hepatitis B and was kindly provided by Dr. Jia Wang (Department of Microbiology, Peking University Health Science Center, China). HBV genomic DNA was isolated using DNAout reagent (Biolife Biotechnology Co., Ltd., Beijing, China), and HBx was

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amplified using primers HBx-up-1 and HBx-down-1 (Table 1). Six repeated His codons were added to primer HBx-down-1 so as to obtain the HBx PCR product with a 6×His tag at the 3′ end. The resulting PCR product was digested with XhoI and HindIII (Takara Biotechnology Co., Ltd., Dalian, China), cloned into pCDNA3.1-B (Invitrogen; abbreviated as PCDB), and sequenced. The resulting plasmid was designated as PCDB-HBx. PCDB and PCDB-HBx were transfected into B16 cells as suggested by Sofast Cell Transfection reagent kit (Biolife Biotechnology Co., Ltd., Beijing, China). Briefly, approximately 2 μg of PCDB-HBx or PCDB was added into 70% confluent B16 cells in culture medium containing Sofast transfection reagent. After incubation for 6 h at 37 °C, cells were resuspended in fresh culture medium without transfection reagent. G418 (Amresco) was added 24 h later at a final concentration of 800 μg/ml to screen for transfected cells. This G418 concentration was maintained for 72 h, and then it was gradually reduced to 100 μg/ml two weeks later. We chose well isolated colonies of cells and transferred them to fresh culture plates to culture them separately. We then verified the effect of transfection through RT-PCR and Western blot. PCDB and PCDB-HBx were transformed into *S. typhimurium* SL3261 by electroporation (Bio-Rad Inc.).

2.3. Construction of plasmids with SspH2-fusions

SspH2 segment with the native promoter (−361 to + 642 nt from start codon) was PCR-amplified from *S. typhimurium* SL1344 using primers NSspH-pro and NSspH-rev (Table 1). Then the PCR product was digested with BglII and EcoRI (Takara Biotechnology Co., Ltd., Dalian, China) and cloned into pET-28a plasmid (Novagen) to generate pET-SspH. To create EcoRI and XhoI ends, HBx was PCR-amplified using PCDB-HBx as the template and HBx-up-2 and HBx-down-2 as primers (Table 1). pET-SspH was then digested with EcoRI and XhoI and ligated to HBx full-length segment to generate pET-NSspH-HBx. All recombinant plasmids were chemically transformed into DH5α and selected by Kanamycin at 50 μg/ml. Plasmids were transformed into *S. typhimurium* SL3261 by electroporation.

2.4. RT-PCR and Western blot

TRIzol reagent was used to extract total cellular RNA from 70% confluent B16/PCDB-HBx and B16/PCDB cells. RT-PCR was performed to examine HBx expression with primers HBx-up-3 and HBx-down-3 (Table 1) by the RT-PCR high-plus kit (TOYOBO Co., Ltd., Osaka, Japan). For Western blots, B16/PCDB-HBx and B16/PCDB cells were lysed with Triton X-100 solution (Sigma), and total proteins were resolved by 12% SDS–PAGE and transferred to PVDF membranes electrophoretically. Fusion proteins were detected using rabbit monoclonal anti-6His antibody (1:1000) purchased from Tiangen Biotech Co., Ltd. (Beijing, China) as a primary antibody. Horseradish peroxidase-conjugated Goat anti-rabbit IgG antibody (1:2000, Beijing Biosynthesis Biotechnology Co., Ltd., China) was used as secondary antibody. Binding was detected by enhanced chemiluminescence. Rabbit β-actin (Beijing Biosynthesis Biotechnology Co., Ltd., China) at 1:000 was used as an internal control.

For detection of SspH2-HBx fusion protein expression and translocation, we co-cultured RAW264.7 cells with *S. typhimurium* SL3261 carrying pET-NSspH-HBx or pET-NSspH as follows. Bacteria carrying corresponding plasmids were cultured with vigorous shaking in LB broth with 0.3 M NaCl and 50 μg/ml Kanamycin at 37 °C for 3 h. The bacteria were then added to RAW264.7 cells that had been starved for 1 h in Hank's balanced salt solution (HBSS) at a multiplicity of infection (MOI) of 30:1. The cells and bacteria were co-cultured at 37 °C for 1 h. After washing for three times with fresh HBSS, the cells were incubated at 37 °C for 30 min in DMEM with 100 μg/ml gentamicin to kill cell-surface attached bacteria. The culture supernatant was removed and the cells were washed

Table 1
Primers used in this experiment

Primer name	Primer sequence
HBx-up-1	5′-CGCCTCGAGATGGCTGCTAGGGTGTGCTG-3′
HBx-down-1	5′-CGCAAGCTTCTAGTGGTGGTGGTGGTGGTGGGCAGAGGTGAAAAAGTTG3′
HBx-up-2	5′-GACGAATTCATGGCTGCTAGGGTGTGCTGC-3′
HBx-down-2	5′-GTCCTCGAGGGCAGAGGTGAAAAAGTTGCA-3′ 5′-CCCGTCTGTGCCTTCTCATC-3′
HBx-up-3	5′-CCAATTTATGCCTACAGCCTCC-3′
HBx-down-3	5′-TCCACCACCCTGTTGCTGTA-3′ 5′-ACCACAGTCCATGCCATCAC-3′
G3PDH-F	5′-GACAGATCTAGTTGCCTGATACGGATGAAAACC-3′
G3PDH-R	5′-GACGAATTCGGTAAGACCTGATTCTCCCAC-3′
NSspH-pro	
NSspH-rev	

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for three times with fresh HBSS. Incubation was continued at 37 °C for 18 h before the cells were collected to extract bacterial and host proteins. Total proteins were quantitated, separated in 10% SDS–PAGE and Western blotted using anti-6His monoclonal antibody as described above.

2.5. Oral immunization of mice with Salmonella carrying recombinant plasmids

Forty female C57BL/6 mice, 6–8 weeks old, were purchased from Experimental Animal Facilities of Peking University Health Science Center (Beijing, China). The mice were divided into eight groups, each containing five mice. Four of the eight groups were used for lactate dehydrogenase (LDH) and ELISPOT assays, each immunized with a *Salmonella* strain carrying either SL3261/PCDB, SL3261/PCDB-HBx, SL3261/pET-NSspH or SL3261/pET-NSspH-HBx; the other four groups were used for tumor challenge experiments. The bacteria carrying SL3261/PCDB and SL3261/PCDB-HBx were cultured in LB broth plus 0.3 M NaCl and Kanamycin (50 μg/ml). Each mouse was orally administrated twice with 10^8 CFU each of the bacterial strains at an interval of 14 days.

2.6. CTL activity assay

Two weeks after the final immunization, three out of the five mice in each of the immune response assay groups were sacrificed and lymphocytes were isolated from the spleens. LDH assay was performed as described [30]. Briefly, RBC-depleted splenocytes (1×10^6 cells/ml) were co-cultured with mitomycin C-treated B16/HBX cells (1×10^6 cells/ml) for 5 days, and then non-attached cells were collected as effector cells. At ratios of Effector cells:Target cells = 20:1 and 80:1, effector cells were incubated with B16/HBx cells (5×10^5 cells/ml) in a 96-well flat-bottom plate at 37 °C for 4 h, followed by centrifugation at 800*g* for 10 min. OD490 of the supernatants was measured with Universal Microplate Reader. The percentage of cytotoxicity was calculated as follows:

$$\%\text{Cytotoxicity} = \left[\frac{OD_{\text{experiment}} - OD_{\text{effector spontaneous}} - OD_{\text{target spontaneous}}}{OD_{\text{target maximum}} - OD_{\text{target spontaneous}}}\right] \times 100$$

$OD_{\text{target maximum}}$ values were measured by treating target cells with NP40 and then examining the OD490 values of the supernatants for the total LDH level released from cytoplasm.

2.7. ELISPOT assay

Two weeks after the final immunization, two out of the five mice in each of the immune response assay groups were sacrificed. Spleen lymphocytes were gathered for IFN-γ ELISPOT assays. Mouse IFN-γ ELISPOT Kit was purchased from U-CyTech Bioscience (Netherlands). The procedure for ELISPOT assays was as described [31] with modifications. Briefly, RBC-depleted mouse splenocytes were co-cultured with mitomycin C-treated B16/HBx cells (1:1 responder:stimulator ratio) with 25 U/ml recombinant mouse IL-2 for 5 days. The reconstituted splenocytes were added to 96-well mixed cellulose plates, which had been pre-coated with anti-IFN-γ antibody. After incubation at 37 °C for 24 h, cells were removed. Following three washes with HBSS, the plates were incubated first with a biotinylated secondary antibody and then with alkaline phosphatase-conjugated streptavidin, followed by addition of freshly prepared AEC substrate buffer. Spots were counted under a dissecting microscope.

2.8. Tumor challenge and immunological protection analysis

Two weeks after the final immunization, each mouse from the tumor challenge groups was inoculated subcutaneously into the right flank with 10^5 B16 cells in 100 μl PBS. During the first week after inoculation, mice were observed for tumor growth by palpation. When the tumor became palpable, the size was measured with a caliper ruler and recorded every day for one week. The volume of tumor was calculated as: [(the maximum length) × (the maximum width)^2]/2. One week after the tumor was first palpable, the mice were sacrificed and weighed, and the tumors were dissected and weighed. Tumor weight index was calculated by the formula: (tumor weight)/(body weight).

2.9. Statistics

A two-tailed Student's *t*-test was used to analyze the inter-group differences in LDH-release assays, ELISPOT experiments, tumor sizes or weight indices. All results were presented as means ± standard error (SE). $p < 0.05$ was considered statistically significant.

3. Results

3.1. Cloning and expression of HBx gene

The HBx gene, PCR-amplified from HBV isolated in a hepatitis B patient, had 91% DNA sequence identity with the NCBI Refseq hepatitis B genome sequence (NC_003977, Fig. 1A). The main differences in HBx predicted protein included a deletion of an 8-amino acid segment near the –COOH end and several single amino acid changes (Fig. 1B), but no apparent changes in identified or predicted antigenic epitopes were found. A histidine tag was added to the 3′-end for detection of protein expression. The HBx-His sequence was

A

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Query  1     ATGGCTGCTAGGGTGTGCTGCCAACTGGATCCTACGCGGGACGTCCTTTGTCTACGTCCC  60
Sbjct  1374  ATGGCTGCTAGGCTGTGCTGCCAACTGGATCCTGCGCGGGACGTCCTTTGTCTACGTCCC  1433

Query  61    GTCGGCGCTGAATCCCGCGGACGACCCGTCTCGGGGCCGTTTGGGGCTCTATCGTCCCCT  120
Sbjct  1434  GTCGGCGCTGAATCCCGCGGACGACCCGTCTCGGGGCCGTTTGGGCCTCTACCGTCCCCT  1493

Query  121   TCTTCGCCTGCCGTTCCGGCCGACCACGGGGCGCACCTCTCTTTACGCGGTCTCCCCGTC  180
Sbjct  1494  TCTTCATCTGCCGTTCCGGCCGACCACGGGGCGCACCTCTCTTTACGCGGTCTCCCCGTC  1553

Query  181   TGTGCCTTCTCATCTGCCGGACCGTGTGCACTTCGCTTCACCTCTGCACGTCGCATGGAA  240
Sbjct  1554  TGTGCCTTCTCATCTGCCGGACCGTGTGCACTTCGCTTCACCTCTGCACGTAGCATGGAG  1613

Query  241   ACCACCGTGAACGCCCTGAACGCCCACCAGGTCTTGCCCAAGGTCTTACATAAGAGGACT  300
Sbjct  1614  ACCACCGTGAACGCCC--A-C-C--A---GGTCTTGCCCAAGGTCTTACACAAGAGGACT  1664

Query  301   CTTGGACTCTCTGCAATGTCAACGACCGACCTTGAGGCATACTTCAAAGACTGTGTATTT  360
Sbjct  1665  CTTGGACTCTCAGCAATGTCAACGACCGACCTTGAGGCATACTTCAAAGACTTTGTGTTT  1724

Query  361   AAAGACTGGGAGGAGTTGGGGGAGGAG-TTA----------CT----A---GGAGGCTGT  402
Sbjct  1725  AAAGACTGGGAGGAGTTGGGGGAGGAGATTAGGTTAAAGGTCTTTGTACTAGGAGGCTGT  1784

Query  403   AGGCATAAATTGGTCTGTTCACCAGCACCATGCAACTTTTTCACCTCTGCCTAA  456
Sbjct  1785  AGGCATAAATTGGTCTGTTCACCAGCACCATGCAACTTTTTCCCCTCTGCCTAA  1838
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B

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Query        MAARVCCQLDPTRDVLCLRPVGAESRGRPVSGPFGALSSPSSPAVPADHGAHLSLRGLPV
Sbjct        MAARLCCQLDPARDVLCLRPVGAESRGRPVSGPFGPLPSPSSSAVPADHGAHLSLRGLPV

Query        CAFSSAGPCALRFTSARRMETTVNALNAHQVLPKVLHKRTLGLSAMSTTDLEAYFKDCVF
Sbjct        CAFSSAGPCALRFTSARSMETTVNA---HQVLPKVLHKRTLGLSAMSTTDLEAYFKDCLF

Query        KDWEELGEEL------LGGCRHKLVCSPAPCNFFTSA
Sbjct        KDWEELGEEIRLKVFVLGGCRHKLVCSPAPCNFFPSA
```

Fig. 1. Sequence comparisons of the cloned HBx gene (Query) with that of reference HBV genome (Subject, Accession No. NC_003977). (A) Nucleotide sequences; (B) amino acid sequences.

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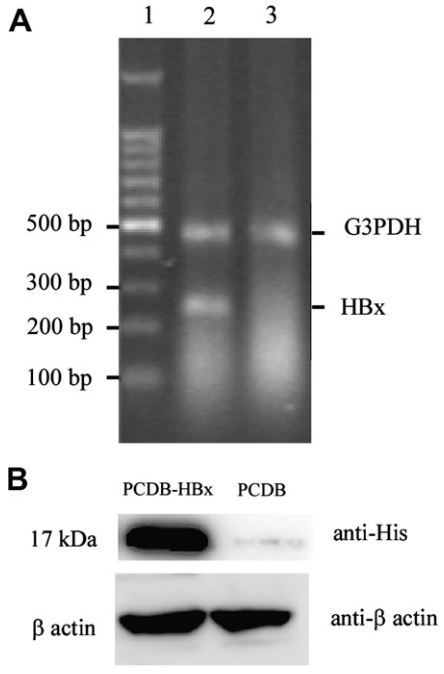

Fig. 2. HBx expression in B16 cells. (A) RT-PCR result of B16 cells stably transfected with PCDB-HBx (lane 2) or PCDB (lane 3). Both HBx and G3PDH-specific primers (Table 1; HBx-up-3 and HBx-down-3 for HBx, product length: 241 bp; G3PDH-F and G3PDH-R for control G3PDH gene, product length: 450 bp) were used in the same reaction. (B) Western blot of B16 cells stably transfected with PCDB-HBx (left) or PCDB (right). The monoclonal antibody (Mab) against His epitope was used for probing HBx recombinant protein. Anti-β actin Mab was used as an internal control.

cloned into plasmid pCDNA3.1-b (PCDB) to generate PCDB-HBx. We transfected PCDB or PCDB-HBx plasmid into B16 cells and confirmed HBx expression by RT-PCR (Fig. 2A) and Western blotting (Fig. 2B). PCDB and PCDB-HBx plasmids were also transformed into live attenuated *S. typhimurium* strain SL3261 for use in animal experiments.

3.2. Expression of NSspH-HBx fusion protein in macrophages

The pET-28a vectors producing NSspH or NSspH-HBx fusion proteins were transformed into the attenuated *S. typhimurium* vaccine strain SL3261. RAW264.7 macrophage cells were infected with the SL3261 bacteria carrying the DNA construct encoding NSspH or NSspH-HBx fusions and analyzed for recombinant protein expression with an anti-6His monoclonal antibody. Western blot results demonstrated that both pellets and supernatants contained a 42 kDa protein (SspH2-HBx fusion protein) 18 h after infection of RAW264.7 macrophage cells by SL3261/pET-NSspH-HBx (Fig. 3).

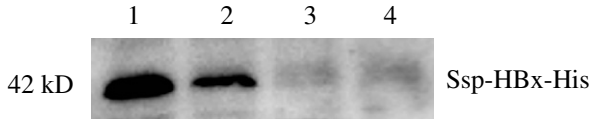

Fig. 3. Western blot detection of SspH2-HBx fusion protein expression under the control of SspH2 native promoter. Representative results of RAW264.7 cells infected with *S. typhimurium* SL3261 vaccine strains carrying pNSspH-HBx (lanes1 and 2) and pNSspH (lanes 3 and 4) are shown. Lanes 1 and 3, cell pellets (Triton X-100 insoluble parts); lanes 2 and 4, supernatant. These results demonstrate that, whereas a major part of the expressed fusion protein SspH-HBx-His seems to be still associated with the bacteria, apparently a significant portion has been delivered to the eukaryotic cells.

3.3. Specific CTL activities against HBx

LDH-releasing assay was performed on spleen lymphocytes to examine the CTL activity elicited by SL3261 carrying the HBx constructs two weeks after final immunization of mice. The spleen cells from mice of the PCDB-HBx-group caused considerable levels of cytotoxicity (Fig. 4A). Similarly, the spleen cells from mice of the pET-NSspH-HBx group also showed significant cytotoxicity (Fig. 4B), although the level of killing was not comparable with that as seen in the PCDB-HBx group ($p < 0.01$ at 20:1 or 80:1 E/T ratio).

3.4. Production of IFN-γ by HBx-immunized mice

ELISPOT assays demonstrated significantly greater numbers of IFN-γ secreting lymphocytes from the spleens of mice immunized with PCDB-HBx (Fig. 5A) or pET-NSspH-HBx (Fig. 5B) than the controls. Like in the CTL activity assays, mice immunized with PCDB-HBx had higher production levels of IFN-γ than those immunized with pET-NSspH-HBx (Fig. 5A and B, $p < 0.05$).

3.5. Tumor inhibition of immunized mice

On the 13th day after tumor challenge, all five mice in each group began to develop palpable tumors, except one in the PCDB-HBx group, which developed tumor on the 14th day. Tumor growth was significantly inhibited in mice immunized with either PCDB-HBx or pET-NSspH-HBx, as reflected in the average tumor growth curves of differently immunized mice (Fig. 6A). On the 19th day after tumor challenge, compared to controls, the tumors on mice of both the PCDB-HBx and pET-NSspH-HBx groups had apparently smaller average tumor volumes (Fig. 6B). Similar trends were observed from the average tumor weight indices on the 19th day (Fig. 6C). We also compared tumor volumes between groups on the 17th and 18th day, and similar results were obtained. Interestingly, a similar magnitude of difference

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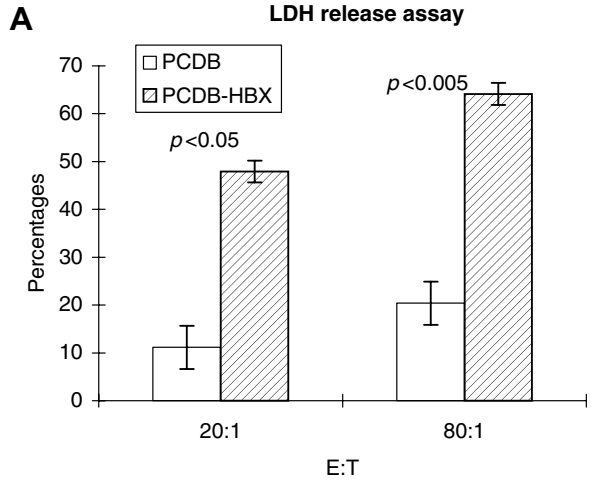


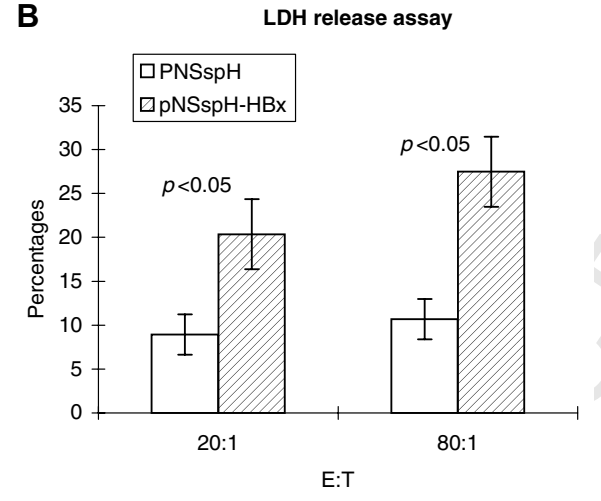
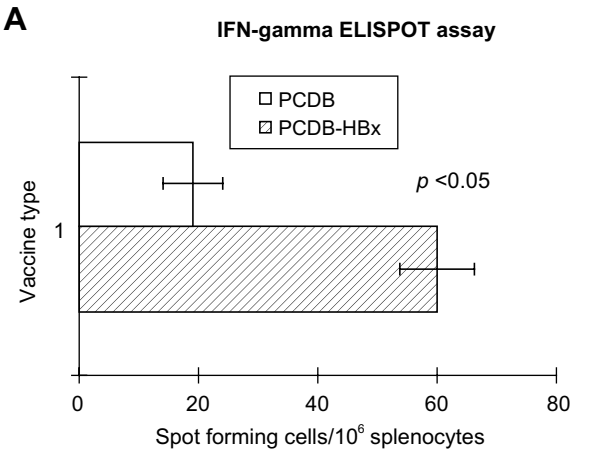


Fig. 4. LDH release assay. (A) LDH release elicited in C57BL mice by SL3261 transformed with PCDB-HBx or PCDB with a 20:1(left) or 80:1(right) of effector to target ratio (effector cells: RBC-depleted mouse lymphocytes; target cells: HBx-expressing B16CL cells). (B) LDH release elicited in C57BL mice by SL3261 transformed with pNSspH-HBx or pNSspH with a 20:1(left) or 80:1(right) of effector to target ratio (effector cells: RBC-depleted mouse lymphocytes; target cells: HBx-expressing B16CL cells).The bars represent different vaccine types and the height of bar represents the average LDH release percentage ($n = 3$) elicited. pNSspH and pNSspH-HBx are the abbreviation of pET-NSspH and pET-NSspH-HBx, respectively.



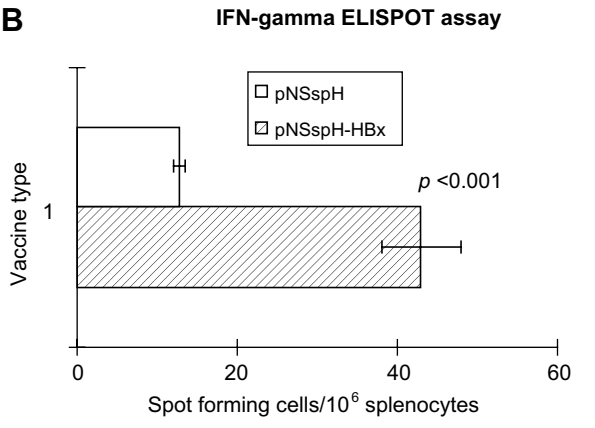


Fig. 5. Mouse IFN-γ ELISPOT assay. (A) Average number of spot forming cells per 10^6 splenocytes of mice immunized with PCDB-HBx vaccine or PCDB control. (B) Average number of spot forming cells per 10^6 splenocytes of mice immunized with pNSspH-HBx vaccine or pNSspH control. pNSspH and pNSspH-HBx are the abbreviation of pET-NSspH and pET-NSspH-HBx, respectively.

seen in CTL and IFN-γ assays between PCDB-HBx and pET-NSspH-HBx groups were not seen for tumor volume or weight index (Fig. 6B and C, $p = 0.10$ and 0.20 for the measurements of tumor volume and weight index, respectively, on 19th day).

We also noticed differences between the vectors: the tumor seemed to grow more slowly in mice vaccinated with PCDB than with pNSspH as judged by tumor volume ($p = 0.04$) or weight indices ($p = 0.0007$). The comparisons were conducted on 19th day after tumor inoculation and all the animals had been immunized and challenged with tumor cells at the same time under the same conditions. One explanation is that PCDB may non-specifically stimulate the immune system, which will need further investigations.

4. Discussion

In this study, we attempted to evaluate the anti-tumor immune effects of two *Salmonella* mediated vaccine strategies. In both strategies, we used HBx as the target epitope, because this viral gene has long been recognized as an important oncogene [23,24] and several CTL epitopic peptides have been

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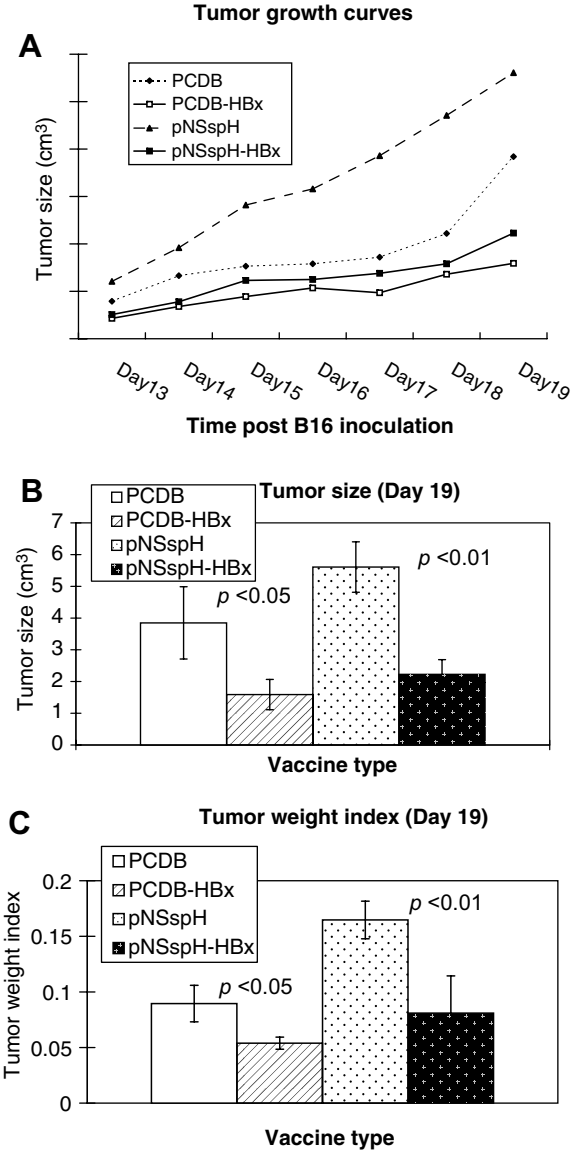

Fig. 6. Mouse melanoma growth inhibition. (A) Tumor growth curves for average tumor volumes ($n = 5$) from Day 13 to Day 19 after inoculation of the mice with B16 cells subcutaneously. The different curves represent tumor growth in mice immunized with different vaccines. (B) Average tumor volumes and (C) average tumor weight index percentages, respectively, of mice immunized with different vaccines on Day 19 after tumor cell inoculation. pNSspH and pNSspH-HBx are the abbreviation of pET-NSspH and pET-NSspH-HBx, respectively.

mapped in the sequence [26,27]. There are reports demonstrating the significant effects of the identified HBx epitopes in eliciting immune responses [28].

By one of the strategies, i.e., using a eukaryotic recombinant plasmid, we constructed PCDB-HBx, which elicited considerable levels of specific immune responses. By the other strategy, i.e., using TTSS for delivering the epitope, we generated a vaccine strain that harbored a vector coding for SspH2-HBx fusion protein. LDH release and ELISPOT assays as well as animal tumor challenging experiments all demonstrated that this SspH2-HBx vaccine could elicit protective immune responses against B16 mouse melanoma cells bearing HBx. In the *in vitro* assays, we noted that immune responses elicited by SspH2-HBx were markedly lower than that elicited by PCDB-HBx. However, we did not see significant differences in immune protection between SspH2-HBx and PCDB-HBx in animal tumor challenging experiments. Further studies involving larger numbers of animals and additional immunological assays may provide insight into this issue.

HBx as a tumor antigen is known to enhance the carcinogenesis and development of hepatoma [23,24]. The antigenicity and even T cell epitopes have been delineated [26–28], opening great opportunities for development of effective vaccines. Unfortunately, however, no HBx-related liver tumor vaccines have been developed. In our experiment, both eukaryotic and prokaryotic HBx recombinant plasmids could elicit significant immune responses against HBx-expressing B16 mouse melanoma cells, demonstrating that both strategies could effectively augment the immunogenicity of the antigen epitopes and may eventually lead to novel vaccines against cancer. Admittedly, the differences between B16/HBx cells and liver cells bearing HBx are extensive. However, our study reported here demonstrated that HBx-expressing mouse tumor cells might be a useful experimental system for evaluating the effectiveness of tumor vaccines. The HBx gene we cloned in this study was slightly different in sequence from those previously published and the main difference lies in loss of a small segment along with a few nucleotide changes in our HBx sequence. Bioinformatics analysis suggests that these nucleotide changes have very little influence, if any, on the overall immunogenicity of HBx protein (data not shown).

In the TTSS-mediated vaccine strategy, we chose reconstructive pET28a plasmid as expression vector because it contains a replicon of moderate-number copies, which ensures the reasonable abundance of antigen protein without apparent toxicity to bacteria [20]. The plasmid contains a 3′-end His tag, which can be used for detection of protein expression. The replacement of the T7 promoter with

SspH2 native promoter region ensured the expression of the recombinant protein.

One of the main concerns in the development of bacteria-mediated vaccines is the stability of antigenic epitope expression. For example, although plasmid-meditated vaccines allow the expression of quite long protein sequences, e.g., PCDB-HBx or pET-NSspH-HBx in this study, with multiple potential epitopes and adjuvant sequences, which may enhance immunological effects, plasmids are usually not stable even though some "balanced plasmids" have been developed [32]. Recently, other groups as well as ourselves are developing a bacterial chromosome-integral vaccines [33,34], in which the stability of vaccine is greatly improved.

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