Vaccine 27 (2009) 1717–1723

Contents lists available at ScienceDirect

Vaccine

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

Recombinant attenuated *Salmonella* harboring 4-1BB ligand gene enhances cellular immunity

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ARTICLE INFO

Article history:
Received 8 November 2008
Received in revised form 10 January 2009
Accepted 12 January 2009
Available online 31 January 2009

Keywords:
4-1BBL
Attenuation *Salmonella*
Cellular immunity

ABSTRACT

Objective: To transfect antigen presenting cells (APCs) with 4-1BB ligand DNA by attenuated *Salmonella enterica serovar* Typhimurium *in vivo*, and to observe the effects of ectogenous 4-1BBL on the immune functions of infected rats.
Methods: Attenuated *Salmonella typhimurium* (vaccine strain) carrying plasmids pIRES2-EGFP-4-1BBL was constructed and used to infect HepG2 hepatoma cells. The expression of reporter gene, green fluorescent protein (GFP) and rat 4-1BBL in the transfected cells was detected by double-immunofluorescence staining. Rats were fed with the recombinant bacteria intragastrically on three occasions in 2 weeks, and were then sacrificed. The transcription and expression of GFP and 4-1BBL genes in splenocytes were measured by RT-PCR and flow cytometry. The phenotypes of T cells in peripheral blood and splenocytes were determined by flow cytometry. The content of IFN-γ in the cultural supernatant of splenocytes stimulated by PHA was measured by ELISA.
Results: The recombinant bacteria harboring 4-1BBL had the same invasive abilities as the original bacteria, and it was able to deliver exogenous genes into HepG2 cells, where the GFP and 4-1BBL were successfully expressed. There were significant upregulations of $CD3^+CD8^+$ T cells ($P=0.018$) and $CD3^+CD25^+$ T cells ($P=0.019$) in the peripheral blood cells as well as $CD3^+CD8^+$ T cells ($P=0.022$), and $CD3^+CD25^+$ T cells ($P=0.008$) in splenocytes of the infected rats. The rats had more 4-1BBL expression detected in the spleen. IFN-γ released by PHA-stimulated splenocytes increased significantly by the recombinant bacteria as compared with controls ($P=0.002$).
Conclusion: Salmonella serovar Typhimurium containing 4-1BBL can transfect target genes into antigen presenting cells *in vivo*, and the expression of exogenous 4-1BBL enhances cellular immunity markedly.
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1. Introduction

4-1BB and 4-1 BB ligands (L) are a pair of important co-stimulatory molecules, acting as second signals to antigen presenting cells (APCs) and T cells [1,2]. 4-1BB ligation enhanced T cell expansion, augments the effector function of T cells [3], and prevents activation-induced apoptosis and functional impairment of murine and human $CD8^+$ T cells [4]. Treatment of dendritic cells (DCs) with anti-4-1BB antibody prevents their presentation of antigens to, or properly priming naïve $CD4^+$ T cells, leading to T cell anergy [20]. 4-1BBL, as the physiological ligand of 4-1BB, has a similar biological function as agonistic 4-1BB antibodies but without the side effects and toxicity of the latter [5]. Using a ligand-based therapy enables the targeting of the co-stimulatory molecules to the tumor site by fusion to tumor-targeting antibodies, thereby avoiding localization to normal tissues [6]. Recombinant poxvirus used as a tumor vaccine, demonstrates that 4-1BBL can cooperate with B7 in enhancing anti-tumor and immunologic responses. However, the effect of the recombinant virus against tumors depends on the ability of vaccine-primed T cells to traffic to the sites of tumor growth and may be inhibited by tumor microenvironment [7]. Therefore exploitation of transfection with DCs may be desirable. However, 4-1BBL could not be effectively expressed *in vivo* to induce the immune response by immunization with naked DNAs encoding tumor antigen and 4-1BBL or RANK/RANKL [8]. Live attenuated *Salmonella* strains that express a foreign antigen have been well studied. Recombinant *Salmonella* strains can be administered via the easy, safe, and well-accepted oral route and can induce strong mucosal and systemic immune responses to the foreign antigen, conferring protective immunity against numerous pathogens in several animal models [9,10].

In this study, we constructed 4-1BBL gene expression plasmids to transform the attenuated *Salmonella*. We demonstrate that the recombinant *Salmonella* strain is an effective vehicle to transfer

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0264-410X/$ – see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.vaccine.2009.01.031

4-1BBL DNA into APCs and over-expression of 4-1BBL can significantly enhance T cell-based cellular immune function, indicating the delivery system may be a prospective method for oral treatment of diseases.

2. Materials and methods

2.1. Materials

Sprague–Dawley (SD) rats (male, aged 6–8 weeks) were obtained from the center of experimental animal of Suzhou University. Plasmid pIRES2-EGFP was purchased from BD Biosciences Clontech (Palo Alto, CA, USA). The recombinant 4-1BBL gene expression plasmid, pIRES2-EGFP-4-1BBL was constructed by our group as described previously [11]. Strains of *Salmonella typhimurium* LB5000 and the attenuated *Salmonella enterica serova typhimurium* with the aroA genetic defect were generously provided by Professor Stocker from Stanford University, USA. Taq DNA polymerase and reverse transcriptase were obtained from TakaRa (Dalian, China). Primers were synthesized by Sangon (Shanghai, China). Fetal calf serum (FCS) and RPMI1640 medium were purchased from GIBCO and PHA-P from Sigma (St. Louis, MO, USA). Antibodies were obtained from each source: goat anti-4-1BBL antibody (CD137L, Santa Cruz) (Santa Cruz, CA, USA); rabbit anti-GFP antibody, donkey anti-goat-CY3 and goat anti-rabbit-FITC antibody (Beyotime Institute of Biotechnology, HaiMen, Jiangsu, China); CD3-PE, CD3-FITC, CD8-FITC (ebioscience), CD4-FITC and CD25-PE (Invitrogen, Carlsbad, CA, USA).

2.2. Construction and identification of attenuated Salmonella containing plasmids

Plasmids pIRES2-EGFP (C) and pIRES2-EGFP-4-1BBL (R) were electro-transfected into salmonella LB5000. After modification, both kinds of plasmids were extracted and electro-transfected into attenuated *S. serovar typhimurium* SL3261. The recombinant bacteria were named SL3261C (SL3261 with pIRES2-EGFP plasmid) and SL3261R (SL3261 with pIRES2-EGFP-4-1BBL plasmid), respectively. They were cultivated in LB-medium (antibiotics free) for 5 days. Both were harvested and specimens were seeded onto LB plates containing kanamycin for screening of positive clones. Polymerase chain reactions (PCR) were applied to identify plasmids extracted from the positive clones, and partial colonies were subjected to Gram staining. In addition, the *Salmonella* A-F polyvalence, O4 thallus and H1 flagella antigens were detected by serological agglutination.

2.3. Invasive power and transfective capability of the recombinant strains in vitro

According to Avogadri's method [12], Single bacteria colonies of SL3261 were cultivated in LB medium, and bacteria colonies of SL3261C, SL3261R were cultivated in LB containing 25 mg/l kanamycin in 37 °C for 18 h. The final concentrations of bacteria were 2.0×10^8 CFU/ml. Infection experiments were divided into four groups: (i) the control, (ii) SL3261, (iii) SL3261C, and (iv) SL3261R. Each group was replicated three times. HepG2 cell monolayers were washed with RPMI1640 medium and then 100 μl of PBS, SL3261, SL3261C, SL3261R, respectively, were added to the wells in 2 ml complete medium containing 50 IU/ml gentamycin. HepG2 cells were collected after cultivating for 24 h, and 1×10^3 cells from each group were lysed in 1 ml sterile purified water. Then, 200 μl of cellular lysates were inoculated onto LB plates and cultured at 37 °C for 18 h for invasive power assay. The remaining HepG2 cells in each group were cultivated with exposure to Ciprobay (2 U/ml) for 24 h and G418 (800 μg/ml) for 2 months. For screening the transfection of the 4-1BBL gene, the stable transfected HepG2 cells were fixed in acetone, stained with goat anti-4-1BBL and rabbit anti-GFP antibodies, washed with PBS and stained with donkey anti-goat-CY3 antibody and then goat anti-rabbit-FITC antibody. The stable transfected HepG2 cells were subjected to RT-PCR for detecting the rat 4-1BBL transcription.

2.4. SD rats were infected by recombinant strains

Male SD rats aged 6–8 weeks were raised at 21 ± 2 °C for 2 days, and then divided randomly into 4 groups, with 5 rats per group. For the group control, rats were gavaged with 1 ml PBS; for the groups SL3261, SL3261C, and SL3261R, rats were intragastrically fed with 10^9 of the respective bacteria in 1 ml suspensions. Water intake was prohibited 2 h before drenches. Salmonella were given to each animal three times on days 3, 10 and 17. Fourteen days after the last dose, all rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p. injections), blood samples were collected from hearts, and then rats were killed. The spleen was removed and one part preserved at −80 °C. The other part of the spleen was grinded under sterile conditions in PBS through a 400 mesh stainless steel cell strainer to prepare cellular suspensions. Mononuclear cells were then isolated via Ficoll-paque density gradient centrifugation, and were washed and resuspended with RPMI1640 medium for the IFN-γ study.

2.5. Detection of report gene GFP in spleen by RT-PCR and flow cytometry

To detect green fluorescent protein (GFP) transcription, total RNA was extracted with the RNAiso Reagent kit (Takara, DaLian, China) and cDNA was generated by reverse transcription of 2 μg of total RNA using random primers and Primescript™ RT Reagent Kit (Takara, DaLian, China) in a total volume of 20 μl, according to the manufacturer's instructions. The sequences of forward and reverse oligonucleotide primers, specific to the chosen candidates and housekeeping genes were 5′-CAC AAG TTC AGC GTG TCC G-3′ and 5′-CTC GAT GCG GTT CAC CAG-3′ for GFP; 5′-GAG GGA AAT CGT GCG TGA C-3′ and 5′-TAG AAG CAT TTG CGG TGC-3′ for β-actin. PCR amplification was carried out in 25 μl of reaction mixture. Initial denaturation at 94 °C for 5 min was followed by 30 cycles of a denaturation step at 94 °C for 30 s, an annealing step at 49 °C for 30 s, and an extension step at 72 °C for 35 s. A final extension step at 72 °C for 5 min was added. After amplification, 5 μl of the reaction mixture was electrophoresed through a 1.5% agarose gel. For the detection of GFP expression in groups SL3261C, and SL3261R, a flow cytometer (Beckman Coulter) was used, and splenocytes of group SL3261 were kept as control.

2.6. Detection of T cell phenotypes

Peripheral blood mononuclear cells (PBMC) and splenocytes were examined for the phenotypes of T cells by flow cytometry, following staining with antibodies. Briefly, cells were stained with antibodies by incubation for 20 min. After the lysis of erythrocytes for 40 min, cell suspension were washed with PBS and re-suspended for flow cytometry. The following commercial antibodies were employed for staining cells: CD3-PE, CD3-FITC, CD8-FITC, CD4-FITC, CD25-PE.

2.7. Detection of rat 4-1BBL expression and IFN-γ production

Frozen sections (6–8 μm) were fixed in paraformaldehyde, blocked and permeabilized in PBS, 3% BSA, 0.1% Triton, then stained with antibodies for 4-1BBL and GFP. Splenocytes from the four groups were cultured in RPMI1640 medium containing 10% FCS,

2 U/ml Ciprobay and 10 μg/ml PHA-P (Sigma) at 37 °C, 5% CO_2 for 72 h. Each group was replicated three times. The supernatants from cultures were collected, and the IFN-γ was measured by enzyme-linked immunosorbent assay (ELISA) using matched-pair antibodies (Jingmei biotech, Shenzhen, Canton, China) according to the manufacturer's instructions.

2.8. Statistical analysis

The results are presented as means ± S.D. The significance of the differences between various treatments was assessed by analysis of variance (ANOVA); comparisons between every two groups were assessed by Tukey HSD post hoc tests. $P < 0.05$ was considered significant.

3. Results

3.1. RT-PCR and morphological identification of the recombinant strains

The electrophoresis results showed the product of RT-PCR of SL3261R was about 930 bp (data not shown), which corresponded with our previous report. SL3261 serum agglutination test showed the A-F polyvalence, and that O4 thallus and H1 flagella antigens were positive in all groups. Morphologically, SL3261 were Gram negative, small and short bacilli; however, SL3261with plasmid was Gram negative trichobacteria (Fig. 1).

3.2. Detection for invasiveness of vaccine strains in HepG2 cells

The colony number of SL3261 strains from 200 disrupted HepG2 cells were: 0/control, 201 ± 46/SL3261, 163 ± 37/SL3261C, 158 ± 32/SL3261R, and there were no significant differences among infection groups ($P > 0.05$). HepG2 cells in groups of control and SL3261 died when exposing to G418; however, under the same condition, HepG2 cells in groups of SL3261C and SL3261R could continue to grow and expressed GFP (Fig. 2). RT-PCR results confirmed the existence of rat 4-1BBL appeared in the groups of SL3261R (data not shown). The expression of GFP and 4-1BBL was detected with double-immunofluorescence staining in recombinant HepG2 cells (Fig. 3).

3.3. Expression of reporter gene GFP and 4-1BBL protein in the spleen

GFP mRNAs were only detected in splenocytes of group SL3261C, and SL3261R, but not in group of control and SL3261. The expression of GFP by flow cytometry was consistent with RT-PCR results (Fig. 4). The 4-1BBL by immunostaining was detected in rats with SL3261R exclusively (Fig. 5).

3.4. Detection of T cell phenotypes

The number of $CD3^+$ T cells in PBMC of the SL3261R group was significantly higher than that of the control, but not in groups of SL3261 and SL3261C. $CD3^+CD8^+$ T cells, but not $CD3^+CD4^+$ T cells in PBMC of the SL3261R group, were significantly higher than that of other groups ($F = 4.479$, $P = 0.018$, Fig. 6A). The results of splenocytes showed that $CD3^+CD8^+$ and $CD3^+CD25^+$ T cells in the SL3261R group were markedly higher than that of other groups ($F = 4.256$, $P = 0.022$ and $F = 5.583$, $P = 0.008$, respectively; Fig. 6B).

3.5. IFN-γ secretion by splenocytes

The production of IFN-γ in the cultural supernatants from splenocytes stimulated by PHA of each group was detected via ELISA. The results showed that there were significantly increased IFN-γ in the SL3261R group than that of other groups ($F = 7.843$, $P = 0.002$). However, there were no differences among other groups ($P > 0.05$) (Fig. 7).

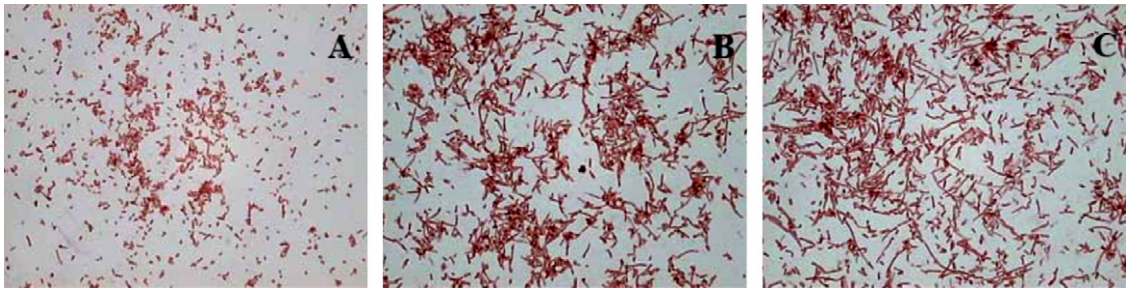

Fig. 1. Morphologyy of *Salmonella typhimurium* and attenuated *Salmonella enterica strains*. The bacteria were stained with Gram staining (1000×). (A) SL3261; (B) SL3261C; (C) SL3261R.

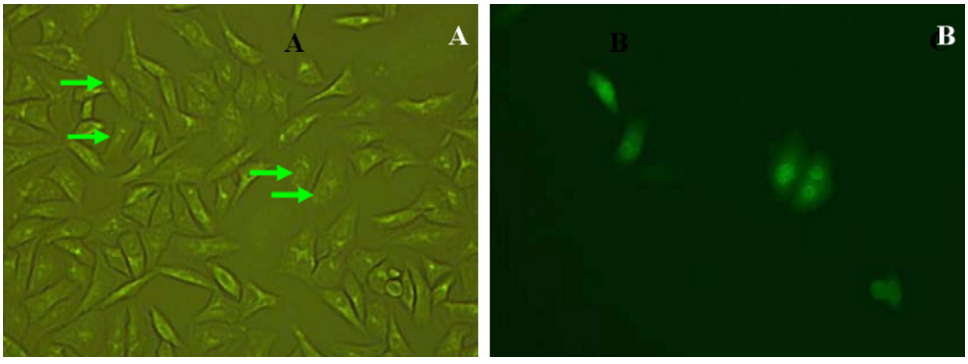

Fig. 2. The invasiveness of SL3261R in HepG2 cells by fluorescence microscopy. (A) In visible light, arrows indicated HepG2 cells, in which GFP expressed. (B) In ultraviolet light (200×).

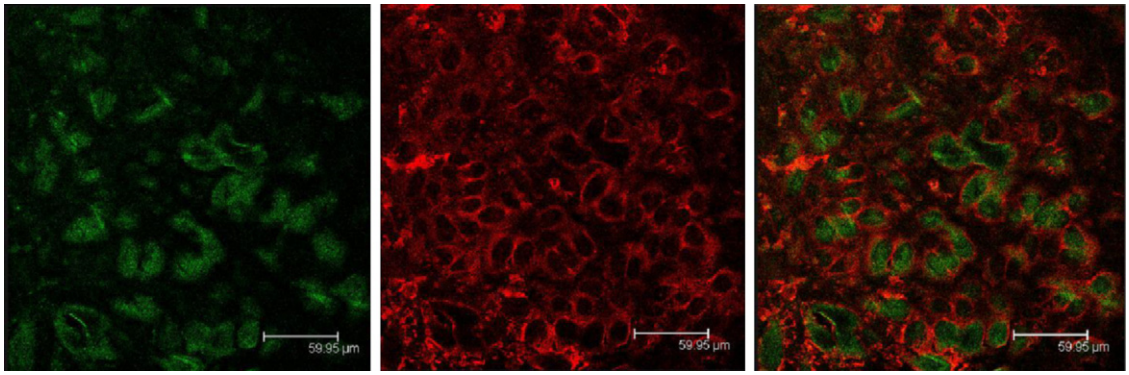

Fig. 3. Detection of rat 4-1BBL on recombinant HepG2 cells. HepG2 cells were incubated with SL3261R for 1 h and eliminated bacteria with gentamycin, 24 h later Ciprobay was used to kill the bacteria inside the cells and then screened by G418 for 2 months. The recombinant HepG2 cells were stained with rat 4-1BBL and anti-goat-CY3 (red) and rabbit anti-GFP and goat anti-rabbit-FITC (green), and both of GFP and rat 4-1BBL (yellow). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

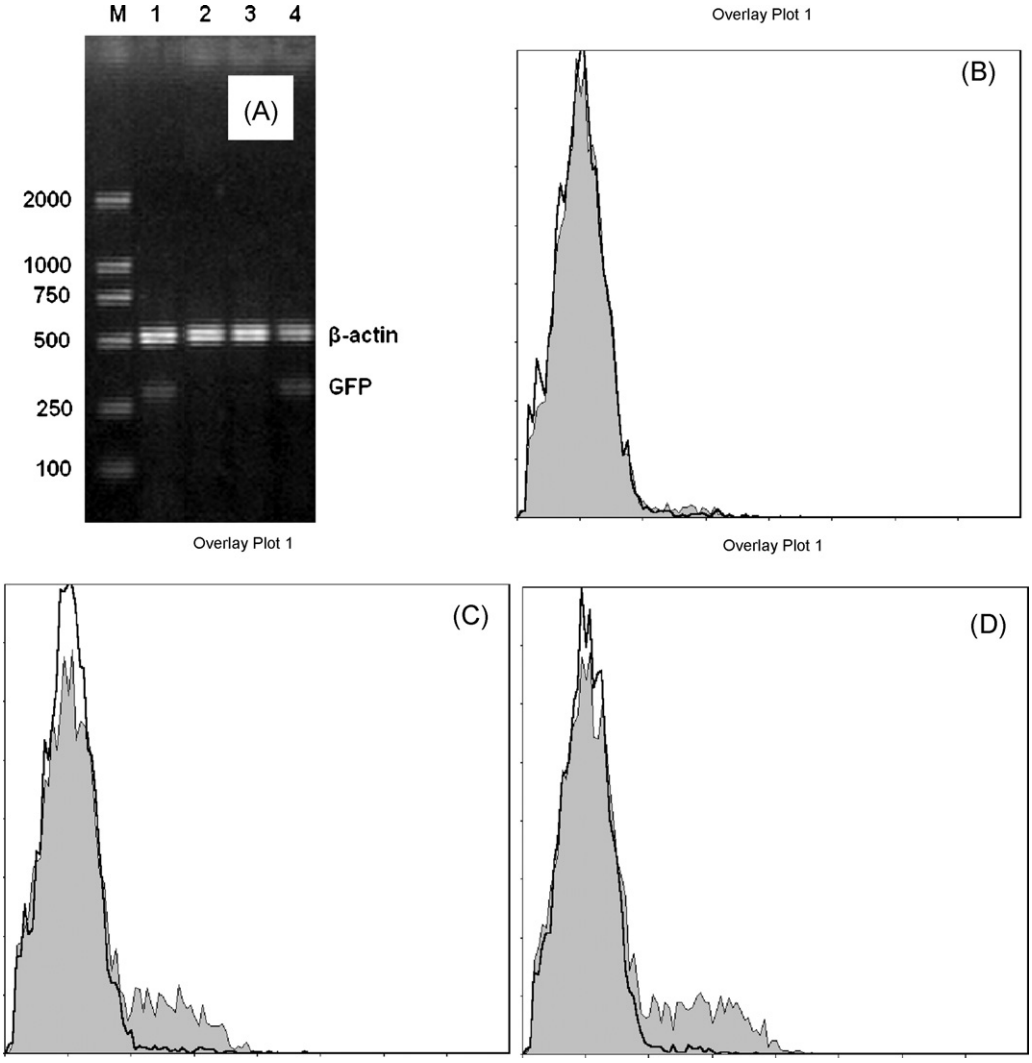

Fig. 4. Transcription and expression of GFP in splenocytes. (A) Transcription of GFP in splenocytes by RT-PCR. M, molecular marker; Lanes 1, and 1 group of SL3261R; 2, control; 3, group of SL3261; 4, group SL3261C. (B), (C) and (D) shows the expression of GFP in splenocytes by flow cytometry. The black line indicated results of control antibody, and shade area in (B), (C) and (D) indicated GFP-positive cells from the groups of SL3261, SL3261C and SL3261 R, respectively.

4. Discussion

Salmonella invade cells by multiple channels. One is the non-epithelial channel into mononuclear macrophages system, such as monocytes, macrophages and DCs, while another one is through type III secretion system secreted by *Salmonella* into the epithelial cells [9]. SL3261 is the aroA-gene mutant strain of *Salmonella*. This genetic mutation leads to nutritional deficiencies of aromatic

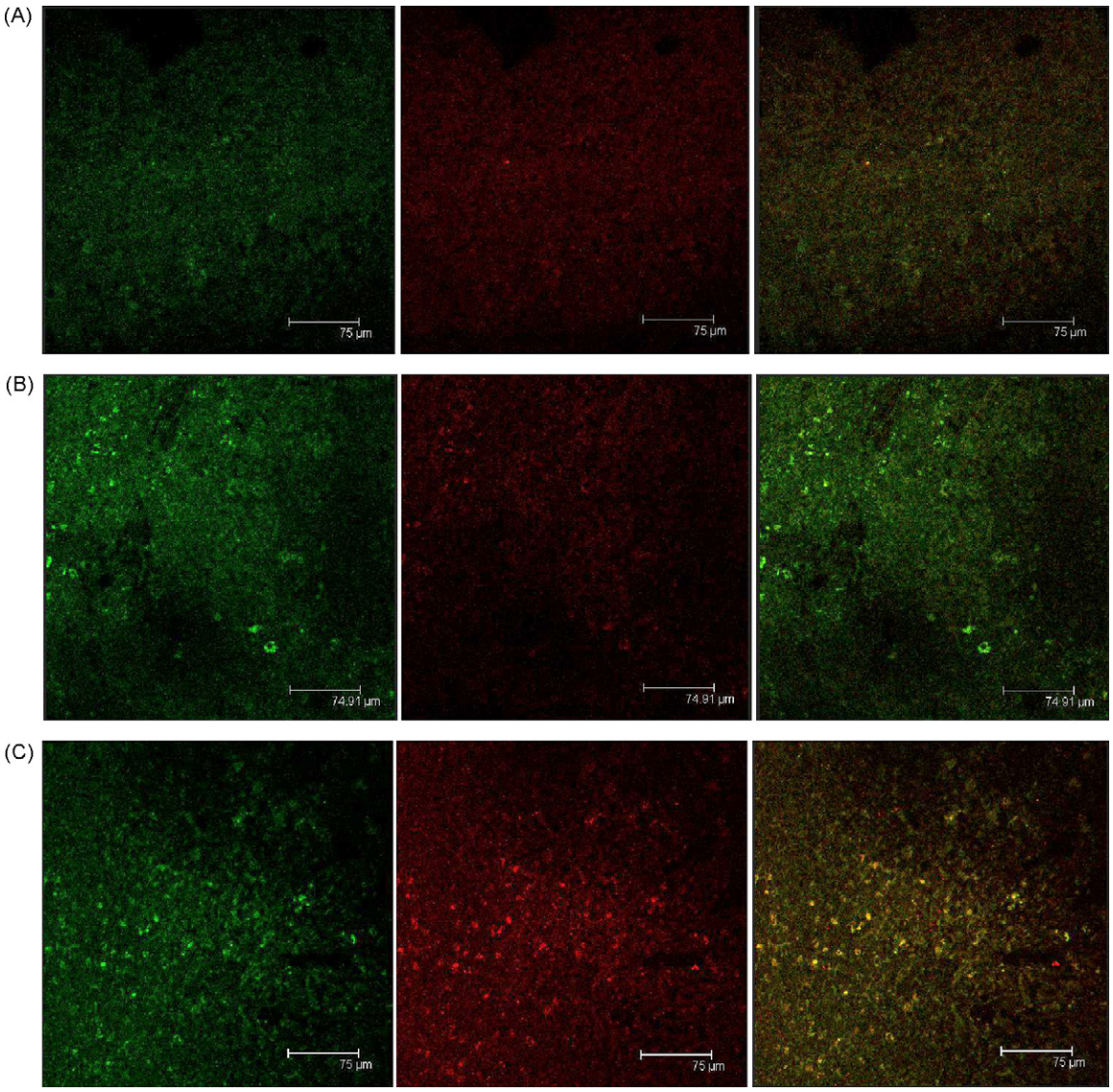

Fig. 5. Detection of 4-1BBL expression on rat spleen. Confocal microscopy was used to analysis the expression of 4-1BBL and GFP of spleen sections. Expressions of 4-1BBL and GFP were made visible with anti-CY3 and anti-FITC antibodies. (A) Rat fed with SL3261; (B) SL3261C; (C) SL3261R.

amino acids, and therefore cannot proliferate in mammalian cells. It has been suggested that plasmids carried by the SL3261 were released and taken up into eukaryocytes after bacteria died [9]. The use of attenuated *Salmonella* as a delivery system eliminates the requirement for DNA purification and allows specific targeting of cells, which has been demonstrated by numerous studies [13,14]. Previously, we used anti-tumor suicide gene carried by SL3261 and obtained good experimental results [15–17]. In the

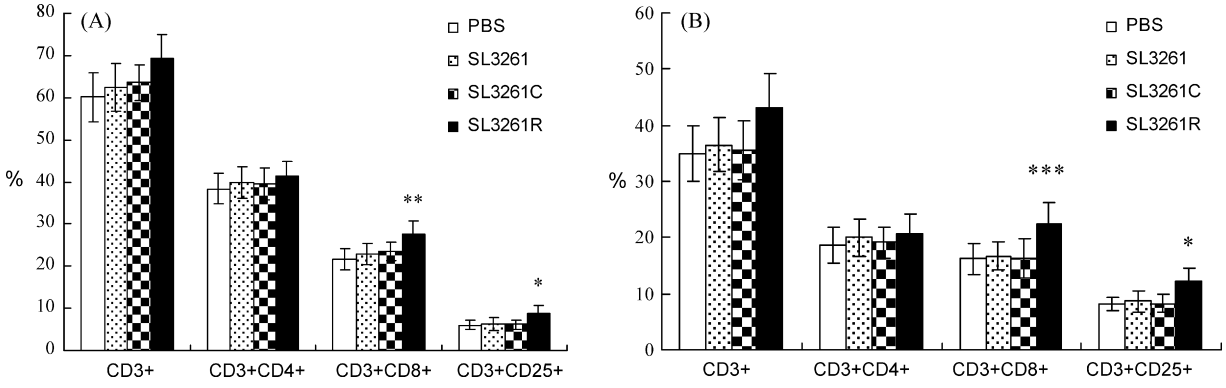

Fig. 6. Phenotypic changes of T cells by vaccination. T cell phenotypes in PBMC (A) and splenocytes. (B) From vaccinated rats was determined by flow cytometry. $^{*}P < 0.05$ as compared with PBS, SL3261 and SL3261C. $^{**}P < 0.05$ as compared with PBS control. $^{***}P < 0.05$ as compared with PBS and SL3261.

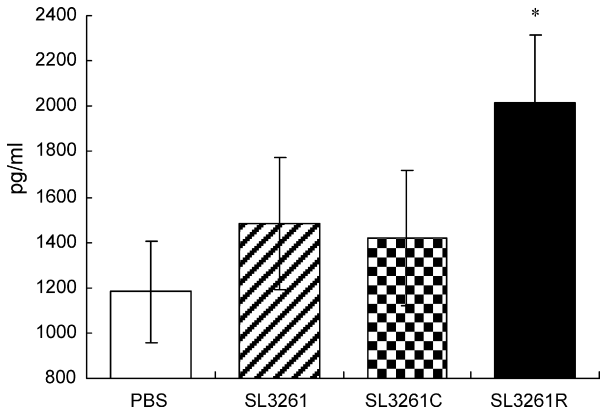

Fig. 7. Production of IFN-γ by splenocytes from vaccinated rats. IFN-γ was determined by ELISA. Supernatants were collected from 72 h of PHA-stimulated splenocytes culture. $^{*}P < 0.05$ as compared with SL3261 and SL3261C; $^{*}P < 0.01$ as compared with PBS control.

present study, attenuated *Salmonella* SL3261 were transformed by a plasmid encoding 4-1BBL gene, and were passaged repeatedly. The serologic tests showed the same results between the transformed strains and the primary strains. However, the morphological features were changed, i.e. the thalline of vaccine strains were filament-like, which resulted in different counting conversion between vaccine strains and primary strains through optical density value. The phenomenon may be due to the fact that only bacteria containing plasmids could produce protein against antibiotics in the kanamycin selection process. The time required for replication of pIRES2-EGFP, which is suitable for engineering the bacteria, is longer than time required for bacteria's division, and leads to filament-like thalline, which in turn ensures growth and survival of bacteria in the selection process. The other probable reason might be that the transduction of plasmid interferes with bacterial metabolism, leading to division obstacles. Our results demonstrated that the recombinant strains and parental strains had similar invasive powers, and the genes carried by the strains could be expressed in the infected cells. The expression of GFP was found in splenocytes of rats infected with SL3261C and SL3261R. Presumably, the ectogenous 4-1BBL genes had been carried into splenocytes by the bacterial delivery system. RT-PCR and flow cytometry confirmed that 4-1BBL was successfully expressed in the spleens. Our results are in agreement with reports of transfected ectogenous genes into DCs by attenuated AroA auxotrophic mutants of *Salmonella typhimurium* (SL3261 and SL7207) [18].

When APCs were transfected with ectogenous 4-1BBL, the costimulation of APCs towards T cells was significantly increased. 4-1BBL can activate T cells, promote clonal expansion and enhance the apoptotic resistance of T cells. Our results showed that the numbers of $CD3^+$ T cells in PBMC increased significantly after infection with recombinant strain SL3261. Most $CD3^+$ T cells were also $CD8^+$ T cells. The results supported the conclusion that 4-1BBL mainly enhances cellar immunity [5–19]. CD25, the receptor of IL-2, expresses predominantly on the surface of activated lymphocytes, and is therefore regarded as a marker of activated lymphocytes. $CD3^+CD25^+$ T cells are regarded as activated T cells. Our results showed that $CD3^+CD25^+$ T cells in PBMC or splenocytes from the group infected with recombinant strain were significantly higher than that of other groups. In our study, the transfection of 4-1BBL by attenuated ST strain into HepG2 cells was like common lipofectamine transfection, and we also used the antibiotic G418 for selecting positive cells. Therefore, the 4-1BBL gene incorporated into immune cells of the living body of a rat cannot self-replicate along with the proliferation of immune cells.

In addition, the higher IFN-γ production in the group also showed that the expression of ectogenous 4-1BBL *in vivo* would lead to a higher state of immunity. However, the therapeutic regimen must be based on a clear understanding of the pathogenesis, the pathogenic cell types involved in the target disease, and the health of the patients selected. Over-expression of 4-1BBL in bodies with multiple health problems might ameliorate one disease while making another worse. For example, 4-1BBL has been reported as a cause of Atherosclerosis [19]. The underlying mechanism is probably that over-expression of 4-1BBL, leading to the autoimune disease by promoting cellular immunity. The over-expression of 4-1BBL can significantly enhance cellular immune function and the bacterial-based delivery system carrying the gene may provide an effective therapy for related diseases. In our studies, we did not find any difference in the degree of Atherosclerosis in rats receiving recombinant strains versus other groups; however, we will analyze the possibility of the side effects of 4-1BBL in the future.

Acknowledgements

We thank Dr. Yao-dong Zhao (Department of Cerebral Surgery, The 2nd Affiliated Hospital, the Laboratory of Aging and Neurodisease, Suzhou University) for his careful review of the manuscript. This work was supported by the Nature Science Foundation of Jiangsu Province of China (BK2008171), the Key Laboratory of Clinical Immunology Foundation of Jiangsu Province, the Innovation Foundation for Graduate Students of Jiangsu Province (2008).

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