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Recombinant salmonella-based 4-1BBL vaccine enhances T cell immunity and inhibits the development of colorectal cancer in rats: in vivo effects of vaccine containing 4-1BBL

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Abstract

Background: Immunotherapy with vaccines is attractive for the treatment of cancer. This study is aimed at determining the effect of recombinant *Salmonella* (SL3261)-based 4-1BB ligand (4-1BBL) vaccine on the development of colorectal cancers and the potential immune mechanisms in rats.

Results: In comparison with that in the PBS group, similar levels of 4-1BBL expression, the frequency of T cells, IFN- γ responses, and comparable numbers of tumors were detected in the SL3261 and SL3261C groups of rats. In contrast, significantly fewer numbers of tumors, increased levels of 4-1BBL expression in the spleens and colorectal tissues, higher frequency of peripheral blood and splenic CD3⁺CD25⁺ T cells, and stronger splenic T cell IFN- γ responses were detected in the SL3261R group of rats.

Conclusion: Our results indicated that vaccination with recombinant attenuated *Salmonella* harboring the 4-1BBL gene efficiently enhanced T cell immunity and inhibited the development of carcinogen-induced colorectal cancers in rats.

Keywords: Recombinant attenuated salmonella, Colorectal cancer, 4-1BBL, T cell-based cellular immunity

Background

Immunotherapy represents an attractive strategy for the prevention of tumor recurrence and the control of cancer growth [1,2]. Immunotherapy can induce antigen-specific T cell responses that can inhibit the growth of tumors. Effective activation of naïve T cells depends on TCR engagement and efficient co-stimulation [3,4]. The 4-1BB ligand (L) is expressed on antigen-presenting cells (APC), and can interact with 4-1BB on activated T cells, co-stimulating T cell cytokine production and proliferation [5-8], and prolonging T cell survival [7,9]. Therefore, 4-1BB and 4-1BBL are important regulators of T cell immunity.

Previous studies have shown that vaccination with antigen peptide linked with 4-1BBL enhances dendritic cell activation and antigen uptake and promotes CD8⁺ T cell

effector/memory responses [10,11]. Similarly, the administration of both 4-1BBL and a HPV-specific E7 peptide or survivin protein eliminates E7-expressing TC-1 cervical cancer or survivin-expressing 3LL lung tumors in vivo [10,11]. Zhang et al. [12] reported that vaccination with natural CD137 ligand, is more efficient and less toxic side effects in inducing colorectal tumor regression in mice, as compared with treatment with anti-4-1BB agonist antibodies. In addition, our previous study has found that vaccination with a recombinant *Salmonella*-based 4-1BBL vaccine efficiently enhances T cell immunity in rats [13]. However, whether and how vaccination with a recombinant *Salmonella*-based 4-1BBL vaccine inhibits the development of colorectal tumors have not been fully illustrated.

In this present study, we investigated the effects of vaccination with a recombinant *Salmonella*-based 4-1BBL vaccine on the development of 1, 2-dimethylhydrazine (DMH)-induced colorectal tumors in rats. Our results

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indicated that vaccination with a recombinant Salmonella-based 4-1BBL vaccine effectively transferred the 4-1BBL gene into APCs and up-regulated 4-1BBL expression in APCs. Moreover, the up-regulation of 4-1BBL expression in APCs induced vigorous T cell immunity and inhibited the development of colorectal cancer in rats.

Methods

Bacteria

Salmonella typhimurium LB5000 and the attenuated Salmonella enterica serova typhimurium SL3261 were generously provided by Professor Stocker from Stanford University, USA. Plasmid pIRES2-EGFP was purchased from BD Biosciences Clontech (Palo Alto, CA, USA). The pIRES2-EGFP-transformed SL3261C or pIRES2-EGFP-4-1BBL-transformed SL3261R were generated, as previously described [13].

Animals

A total of 20 male Sprague Dawley (SD) rats at 6–8 weeks of age were obtained from the Center of Experimental Animal of Soochow University. Rats were housed in a specific-pathogen-free (SPF) facility in 12-h light/dark cycle at $50 \pm 10\%$ humidity and $21 \pm 2^\circ\text{C}$ with free access to water and food. The body weights of individual rats were measured weekly. The experimental protocols were approved by the Ethics Committee of the First Affiliated Hospital of Soochow University.

Establishment of rat model of colorectal tumor

Individual animals were administered subcutaneously with 20 mg/kg 1, 2-dimethylhydrazine (DMH, Sigma, St. Louis, USA) weekly for 20 consecutive weeks. Fourteen weeks after the first DMH administration, the rats were randomized and gavaged with 2 ml PBS, PBS containing 2×10^9 SL3261, the pIRES2-EGFP-transformed SL3261, or the pIRES2-EGFP-4-1BBL-transformed SL3261 every two weeks for three times as the control, SL3261, SL3261C, or SL3261R group, respectively. At 21 weeks post the first DMH injection, the rats were anesthetized and their blood samples were collected and sacrificed. Their spleens were dissected, and a portion of the spleen from each rat was used for immunofluorescent staining.

Gross and histological examination

The entire colorectal (from the cecum to the anus) tissues of individual rats were removed, washed thoroughly with ice-cold saline, cut longitudinally, and laid flat on a board. The numbers of tumors were counted in a blinded manner. The disease stages of colorectal cancers were evaluated by Duke's stage system [14] with minor modifications. Briefly, Stage A: tumors only in the mucosa; Stage B-C, tumors invading into muscularis

propria, but without distant metastasis; and Stage D: tumor with distant metastasis.

Flow cytometric analysis

Peripheral blood mononuclear cells (PBMC) were isolated either at 16 weeks or 21 weeks post the initial DMH administration and splenic mononuclear cells were prepared at 21 weeks post the initial DMH administration by Ficoll-paque density gradient centrifugation. The frequency of different subsets of T cells was characterized by flow cytometry. Briefly, 1×10^5 cells were stained with anti-CD3-PE, anti-CD3-FITC, anti-CD8-FITC, anti-CD4-FITC, and anti-CD25-PE antibodies (Ebioscience), and after washing, the cells were subjected to flow cytometry and analyzed by FlowJo software.

Reverse transcription-polymerase chain reaction (RT-PCR)

The relative levels of GFP mRNA transcripts in splenocytes were determined by RT-PCR. Briefly, total RNA was extracted from individual splenic mononuclear cell samples and reversely transcribed into cDNA using a specific kit TakaRa (Dalian, China), according to the manufacturers' instruction. The sequences of primers were forward 5'-CACAAAGTTCAGCGTGTCCG-3' and reverse 5'-CTCGATGCGGTTACCAG-3' for GFP; forward 5'-GAGGGAAATCGTGCGTGAC-3' and reverse 5'-TAGAAGCATTTGCGGTGC-3' for β -actin. PCR amplification was performed in duplicate at 94°C for 5 min and subjected to 33 cycles of 94°C for 30 s, 49°C for 30 s, and 72°C for 35 s, followed by 72°C for 10 min.

Immunofluorescence

The levels of 4-1BBL and GFP expression were detected by immunofluorescence, as previously described [13]. In brief, the frozen spleen and colorectal tissue sections were fixed in Acetone and stained with goat anti-4-1BBL antibodies (CD137L, Santa Cruz Biotech, Santa Cruz, USA) and rabbit anti-GFP antibodies, respectively. After being washed, the bound antibodies were detected with donkey anti-goat-CY3 and goat anti-rabbit-FITC antibodies (Beyotime Institute of Biotechnology, Haimen, Jiangsu, China). Subsequently, the sections were mounted and examined under a confocal microscope (Leica, Germany).

Enzyme-linked immunosorbent assay (ELISA)

Splenic mononuclear cells were prepared at 21 weeks post the initial DMH administration by Ficoll-paque density gradient centrifugation. Splenic mononuclear cells (2.5×10^5 cells/ml) were stimulated in triplicate with $10 \mu\text{g/ml}$ of PHA-P (Sigma) in RPMI1640 (GIBCO) medium containing 10% FCS and $2 \mu\text{g/ml}$ of Ciprobay (HealthCare, AG) for 72 hours. The supernatants of cultured cells were harvested and the concentrations of

IFN- γ in the supernatants were measured by ELISA using a rat IFN- γ detection kit, according to the manufacturers' instruction (Jingmei Biotech, Shenzhen, China).

Statistical analysis

Data were expressed as the means \pm SD. The difference among different groups was assessed by analysis of variance (ANOVA) and the difference between two groups was assessed by Student's *t*-test. A P value of < 0.05 was considered statistically significant.

Results

Expression of reporter gene GFP and 4-1BBL protein

We first evaluated the reporter gene GFP mRNA transcription in splenocytes of different groups of rats using RT-PCR technique at 21-week post the initial DMH administration. We detected GFP mRNA transcripts in the splenocytes from the rats vaccinated with SL3261C or SL3261R (recombinant attenuated *Salmonella typhimurium* carrying pIRES2-EGFP or pIRES2-EGFP-4-1BBL plasmids), but not in the control and SL3261-vaccinated rats (Figure 1). Further immunofluorescent analysis revealed that GFP protein expression was detected in the spleen and colorectal tissues from the SL3261C- and SL3261R-vaccinated rats (Figures 2 and 3). In addition, analysis of the 4-1BBL expression revealed that the intensity of anti-4-1BBL staining increased in the spleen and colorectal tissues from the SL3261R-vaccinated rats. These observations indicated that vaccination with the SL3261R effectively enhanced 4-1BBL expression in these tissues of rats.

Vaccination with the SL3261R reduces the numbers of colorectal tumors in rats

Following the induction of colorectal tumors, one out of 20 rats died at 20 weeks post the initial DMH treatment. Characterization of animals indicated that a large amount of bloody ascites was found in the rats. A great number of tumor nodules varying in size were observed in the mesentery and posterior peritoneum. The rigor of the colorectal rectal wall appeared. A total of 18 adenocarcinoma were identified by histological examination using H & E staining and visualized in the entire colorectal of the dead rats (data not shown). In addition, the numbers of tumors at 21 weeks post DMH treatment were 18.2 ± 4.7 in the PBS group; 12.8 ± 4.5 in the SL3261-treated group; 12.2 ± 4.4 in the SL3261C-treated group; and 9.4 ± 4.3 in the SL3261R-treated group (Figure 4A). The number of tumors in the SL3261R-treated group of rats was significantly less than that in the PBS group. In addition, we found that most of the tumors in the PBS group of rats were at Dukes' stage D (stage B-C, 1; Stage D, 4), while most tumors in the SL3261 and SL3261C groups were at stage B-C (SL3261: stage A, 1; stage B-C, 2; Stage D, 2; SL3261C: stage B-C, 4; stage D, 1). However, the tumors in the SL3261R group of rats were at stage A-C (Stage A, 2; Stage B-C, 2) (Table 1). Moreover, there was no significant difference in the rat body weights among the four groups, although slightly increased body weights were observed in the SL3261R-vaccinated group of rats 16 weeks after DMH application (Figure 4B).

T cell phenotypes

To understand the anti-tumor effect, the frequency of different subsets of peripheral and splenic T cells was

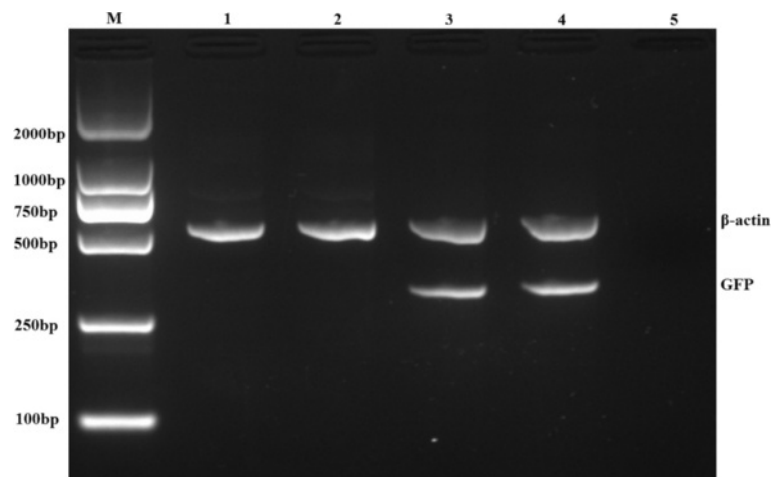


Figure 1 RT-PCR analysis of GFP mRNA transcripts in splenocytes of the rats from different groups. Following administration of DMH for 21 weeks, the rats were sacrificed and their splenocytes were isolated. Subsequently, the GFP mRNA transcripts in splenocytes from different groups of rats were evaluated by RT-PCR. β -actin was used as an internal control. Data shown are representative images from three separate experiments. Lane M, DNA marker; 1, PBS; 2, SL3261; 3, SL3261C; 4, SL3261R; 5, Negative control (RNA without reverse transcription).

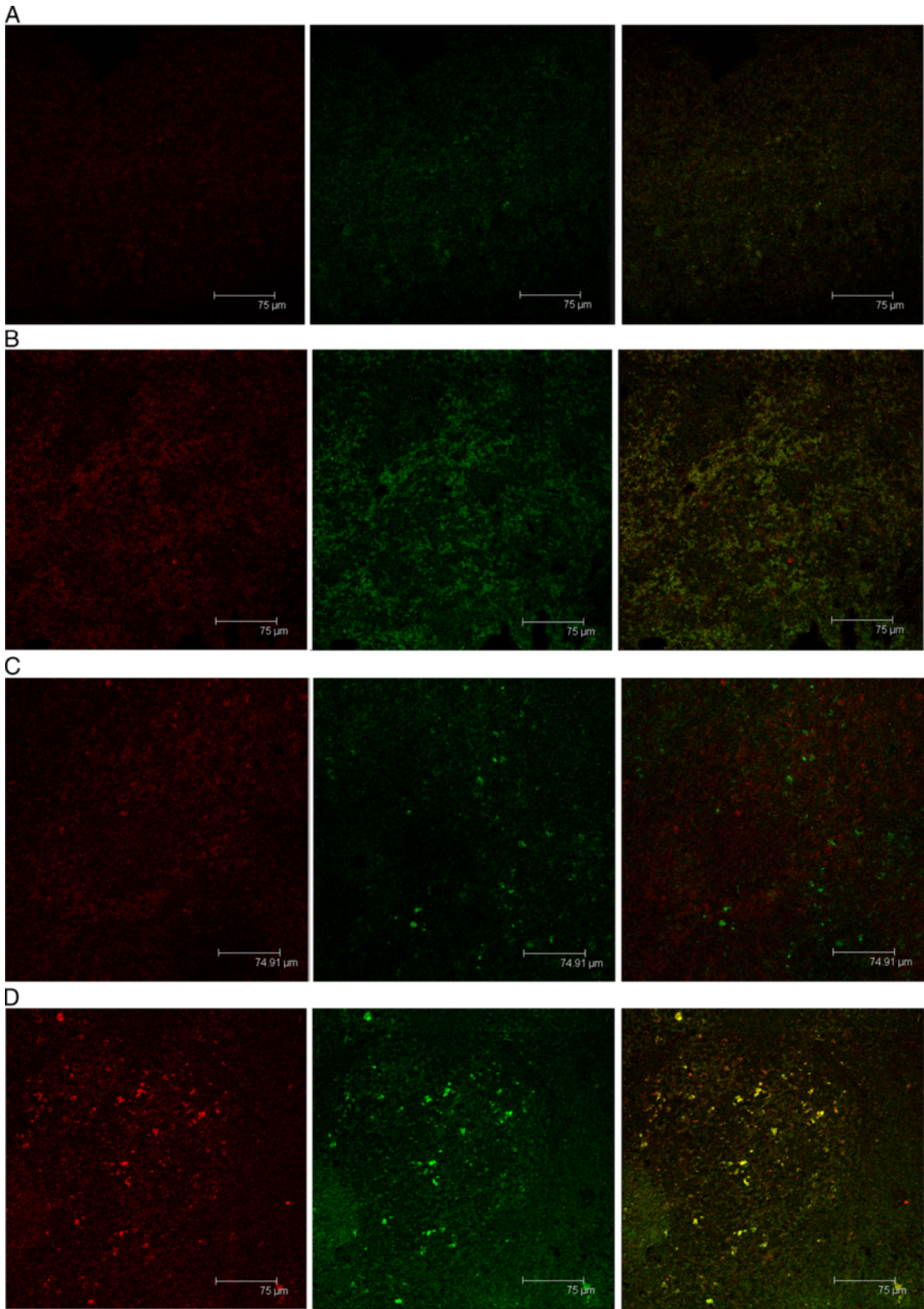


Figure 2 Immunofluorescent analysis of 4-1BBL expression on rat spleens. The expression of GFP and 4-1BBL in the spleens of different groups of rats was characterized by immunofluorescent assays using anti-4-1BBL (red) and anti-GFP (green) antibodies and examining under a confocal microscopy. Data shown are representative images of different groups of rats (n = 4 for the PBS group, n = 5 for other groups) from three separate experiments. **(A)** The PBS group; **(B)** The SL3261 group; **(C)** The SL3261C group; **(D)** The SL3261R group.

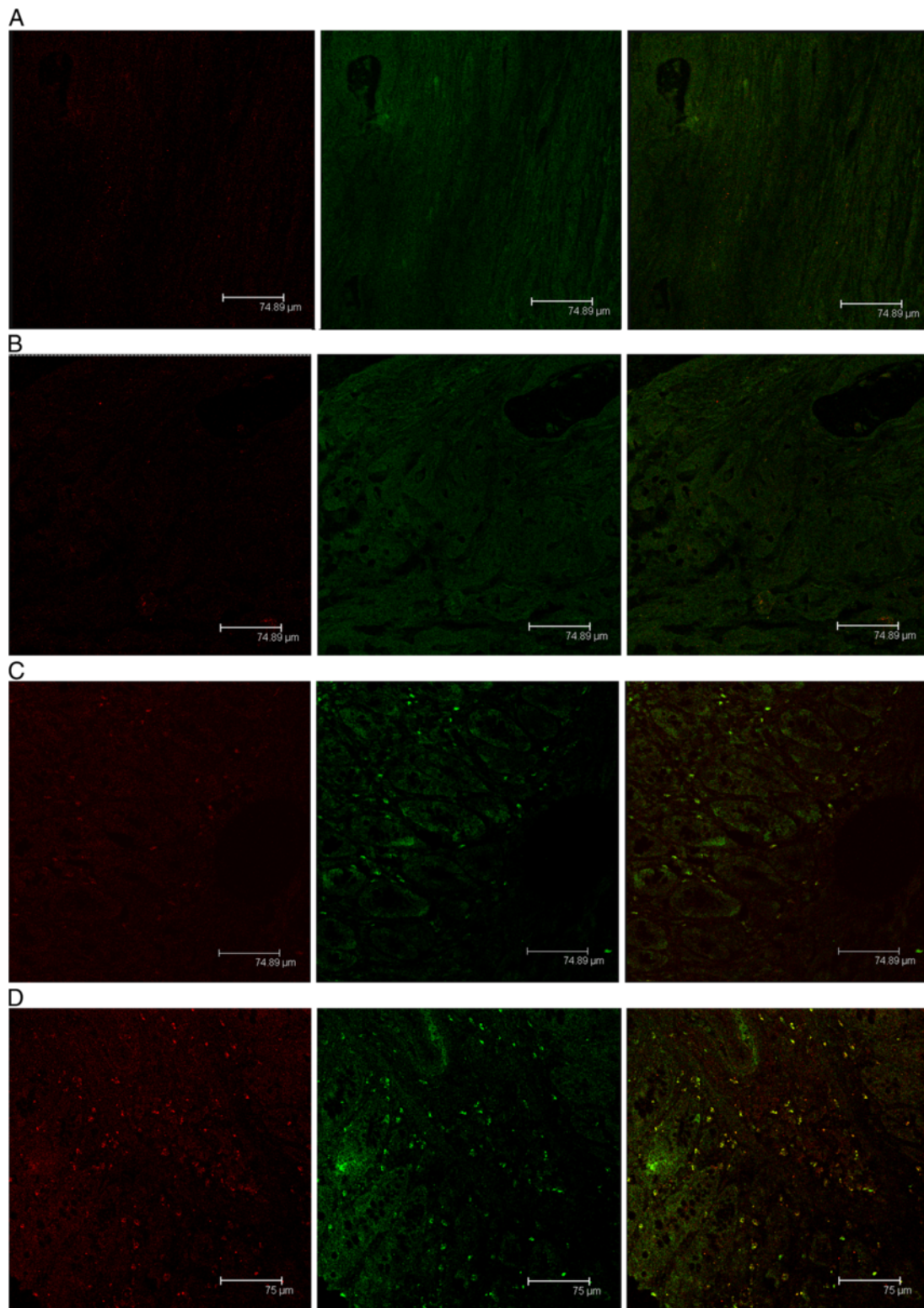
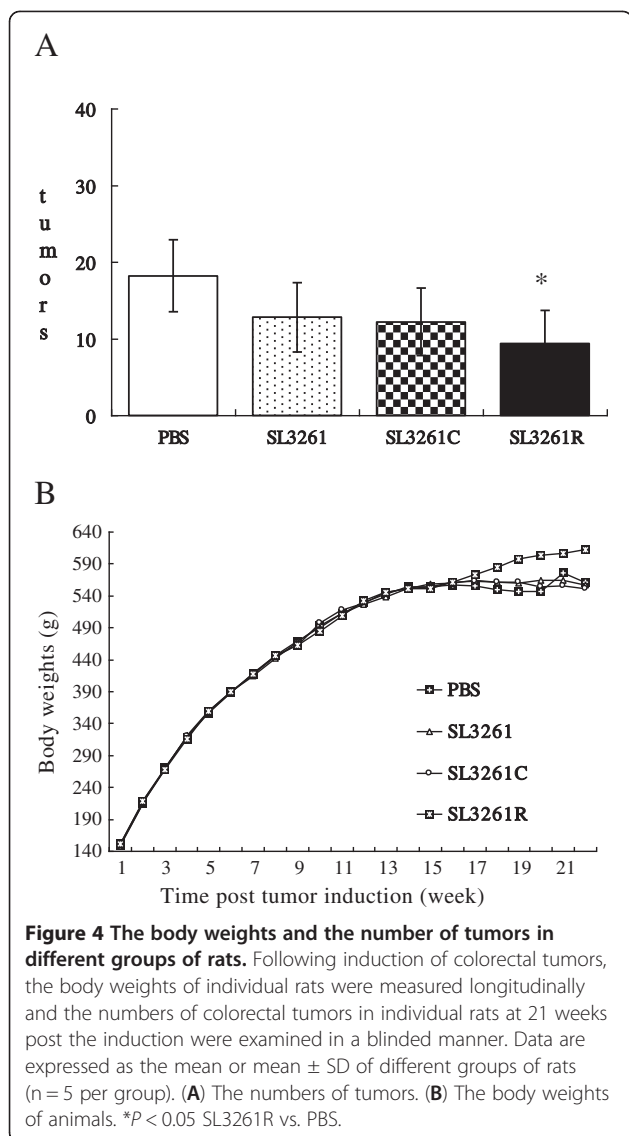


Figure 3 Immunofluorescent analysis of 4-1BBL expression on the colorectal tumor tissues of rats. The expression of GFP and 4-1BBL in the colorectal tumor tissues from different groups of rats was characterized by immunofluorescent assays using anti-4-1BBL (red) and anti-GFP (green) antibodies and examining under a confocal microscopy. Data shown are representative images of different groups of rats (n = 4 for the PBS group, n = 5 for other groups) from three separate experiments. (A) The PBS group; (B) The SL3261 group; (C) The SL3261C group; (D) The SL3261R group.



characterized by flow cytometry analysis at 16 weeks post the initial DMH administration. We found that the frequency of peripheral blood CD3⁺CD8⁺ and CD3⁺CD25⁺ T cells, but not the total CD3⁺ and CD3⁺CD4⁺ T cells, in the SL3261R group was significantly higher than that in the PBS control (P < 0.05) (Figure 5A). No significant difference was found in the number of CD3⁺, CD3⁺CD4⁺

Table 1 Analysis of tumor staging in rats from different experimental groups

Groups	Negative	Stage A	Stage B-C	Stage D
PBS	0	0	1	4
SL3261	0	1	2	2
SL3261C	0	0	4	1
SL3261R	1	2	2	0

The number of animals in Dukes Stage A, Stage B-C or Stage D was listed.

and CD3⁺CD8⁺ T cells of peripheral blood and splenic T cells among PBS control, SL3261 and SL3261C groups 21 weeks after the initial DMH administration (P > 0.05). But CD3⁺CD25⁺ T cells in the SL3261R group was significantly higher than that in the other groups (P < 0.05) (Figure 5B-C).

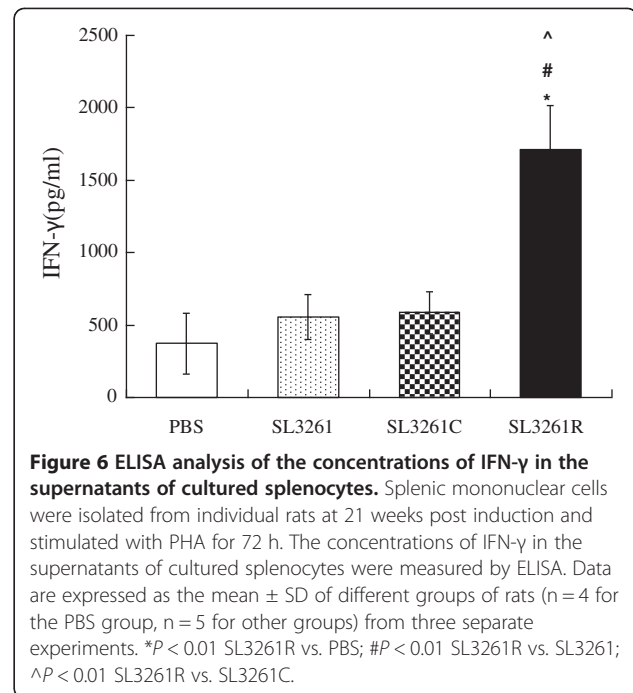
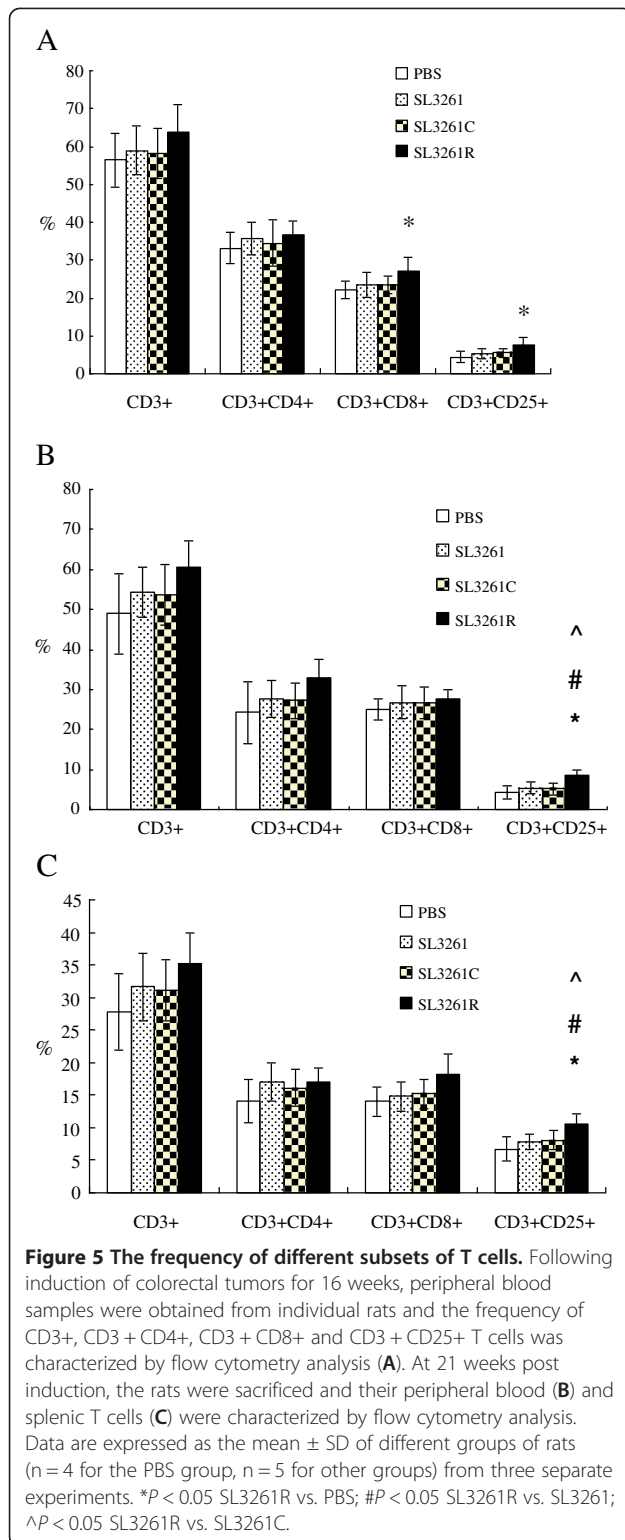
Vaccination with the SL3261R enhances splenic IFN- γ responses

The concentrations of IFN- γ in the cultured supernatants of splenocytes stimulated with PHA from individual groups of rats were analyzed by ELISA. As shown in Figure 6, the levels of IFN- γ were comparable among the PBS, SL3261, and SL3261C-treated rats. In contrast, the concentrations of IFN- γ in the cultured splenocytes from the SL3261R-vaccinated rats were significantly higher than that in the other groups of rats (P < 0.01). However, no significant difference was detected among PBS, SL3261, and SL3261C groups (P > 0.05).

Discussion

The 4-1BBL is a type II surface glycoprotein of the tumor necrosis factor (TNF) superfamily, and is generally expressed in APCs, such as dendritic cells, macrophages, and activated B cells [4]. 4-1BBL binds to 4-1BB (also called CD137), a member of the TNFR superfamily, and enhances T cell activation [4]. Emerging lines of evidence shows that activation of 4-1BB by agonistic monoclonal antibodies (Abs) or over-expressing 4-1BBL enhances T cell proliferation and cytokine production, up-regulates anti-apoptotic gene expression and prevents activation-induced cell death [15-17]. However, systemic administration of anti-4-1BB agonistic Abs is unsuccessful in correcting the defect in response to severe influenza in 4-1BBL-deficient mice, which is possibly because anti-4-1BB can target many cell types and lead to high levels of cytokine production and immunopathology [18,19]. A HIV-1 effective vaccine should induce both antigen-specific cellular and humoral immunity and DNA vaccine for inducing 4-1BBL expression is a superior adjuvant to enhance HIV-specific immunity than anti-4-1BB agonistic antibody [20]. Based on these findings, our previous study has shown that recombinant attenuated Salmonella harboring the 4-1BBL gene can induces high levels of 4-1BBL expression in dendritic and other immune cells [13]. In the current study, we explored the effect of oral treatment with a recombinant Salmonella-based 4-1BBL vaccine on the development of DMH-induced colorectal cancers in rats and the potential immune mechanisms.

A previous study indicated that abnormal morphology characterized by accumulation of undifferentiated cells with pleiomorphic and conspicuous nucleoli in a small cluster of neighboring crypts was found at 12 weeks post DMH administration. Microscopic carcinomatous foci



were observed at 15 weeks post DMH treatment [21]. Therefore, in this present study, the vaccine administration was started 14 weeks after the DMH treatment.

Inoculation of recombinant Salmonella appeared to induce more 4-1BBL expression in the spleens and colorectal tumor tissues. Evidentially, we detected the reporter GFP mRNA transcripts and protein expression only in the spleens from the SL3261C and SL3261R groups of rats. Similarly, there was more 4-1BBL expression in the spleens and colorectal tissues than the other groups. In this study, we found that SL3261R suppressed tumor growth as compared with PBS group (P < 0.05). However, no significant difference was found between SL3261R group and SL3261 or SL3261C group (P > 0.05), although slightly decreased number of colorectal tumors was observed in SL3261R group. As Salmonella can localize to transplantable murine tumors and partially inhibit tumor growth [22], it is possible that the attenuated Salmonella (SL3261) itself may have anti-tumor effect. We further staged the colon cancer and found that most of the tumors in PBS groups were in late stage (Dukes' stage D). In SL3261 and SL3261C groups, tumors were found in middle and late stages (Dukes' stage C D), whereas tumors in SL3261R group were in early and middle stages (Dukes' stage A B C). Together, our data extended previous findings and indicated that recombinant bacteria-based 4-1BBL vaccines effectively inhibited the development of colorectal cancer [12].

CD3⁺CD8⁺ cells are major players of T cell immunity against tumors [23]. CD25 is the α -chain of IL-2R, which

is absent on naive resting T cells, except for T regulatory cells. Following stimulation by “signal two”, activated T cells initiate IL-2 expression and secretion, which acts as an autocrine growth factor because activated T cells express high-affinity IL-2R. IL-2 exerts its pleiotropic activities through binding to either dimeric receptor composed of β -chain IL-2R (CD122) and common cytokine receptor γ -chain (CD132) or the receptor composed of trimeric IL-2R. α ; IL-2R β , and common cytokine receptor γ -chain. CD25 has been considered as a marker of activated lymphocytes [24,25]. Consistent with our previous results [13], the number of CD3⁺CD8⁺ and CD3⁺CD25⁺ T cells in peripheral blood obtained from SL3261R group was increased following induction of colorectal tumors for 16 weeks, when animals were given drug administration for once ($P < 0.05$ compared with PBS group). Nevertheless, no statistical difference was found between SL3261R group and SL3261 or SL3261C group ($P > 0.05$), which might be due to the short time period of drug administration and only once vaccine administration. Significant elevated number of CD3⁺CD25⁺ T cells in peripheral blood was detected 21 weeks following tumor induction, when animals were given 3 times of drug administration in SL3261R group ($P < 0.05$ compared with all groups), suggesting the recombinant bacteria-based 4-1BBL vaccines can enhance the immune function of tumor-bearing mice. We did not observe significant difference in the frequency of peripheral blood and splenic CD3⁺CD8⁺ T cells between the SL3261R and other groups of rats at 21 weeks post DMH treatment. It is possible that CD3⁺CD8⁺ T cells may migrate into the tumors with the progression of tumorigenesis. More importantly, we detected significantly higher levels of splenic IFN- γ responses in the SL3261R group of rats. Given that IFN- γ is predominantly secreted by Th1 and cytotoxic CD8⁺ T cells (the stronger IFN- γ responses), together with a higher frequency of activated T cells suggest that the recombinant vaccine may enhance T cell immunity that inhibits the development of colorectal cancers in rats [26].

Conclusions

In summary, our data indicated that recombinant Salmonella-based 4-1BBL vaccine induced 4-1BBL expression in immune cells and administration of the vaccine efficiently inhibited the development of DMH-induced colorectal tumors in rats, accompanied by increasing the frequency of activated T cells and splenic IFN- γ responses. Our findings may provide a new basis for the design of immunotherapies for the intervention of colorectal cancer.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

JXY and YTZ performed research; JXY and LL wrote the manuscript and analyzed data; DMR and XGZ performed the partial experiments; JXY and WCC conceived of the study and designed research. All authors read and approved the final manuscript.

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Highlights

Recombinant Salmonella-based 4-1BBL vaccine effectively enhanced 4-1BBL expression.

This Vaccine inhibited the development of colorectal cancer in rats. Vaccine enhanced T cell immunity in rats.

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