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Original Article

The treatment of mouse colorectal cancer by oral delivery tumor-targeting *Salmonella*

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Abstract: Systemic administration of *Salmonella* to tumor-bearing mice leads to its preferential accumulation in tumor sites, the enhancement of host immunity, and the inhibition of tumor growth. However, the underlying mechanism for *Salmonella*-induced antitumor immune response via oral delivery remained uncertain. Herein, we used mouse colorectal cancer (CT26) as tumor model to study the therapeutic effects after oral delivery of *Salmonella*. When orally administered into tumor-bearing mice, *Salmonella* significantly accumulated in the tumor sites, inhibited tumor growth and extended the survival of mice. No obvious toxicity was observed during orally administered *Salmonella* by examining body weight and inflammatory cytokines. As indoleamine 2, 3-dioxygenase 1 (IDO) is a crucial mediator for tumor-mediated immune tolerance, we examined the expression of IDO. We demonstrated that *Salmonella* inhibited IDO expression in mouse cancer cells. Furthermore, immunohistochemical studies of the tumors revealed the infiltration of neutrophils and T cells in mice treated with *Salmonella*. In conclusion, our results indicate that *Salmonella* exerts its tumoricidal effects and stimulates T cell activities by inhibiting IDO expression. Oral delivery of *Salmonella* may, represent a potential strategy for the treatment of tumor.

Keywords: Colorectal cancer, tumor-targeting, *Salmonella*, oral delivery, indoleamine 2, 3-dioxygenase 1, T cell

Introduction

The use of *Salmonella* as an oncolytic agent is one of the innovative approaches for the treatment of cancer. This is based on the observation that *Salmonella* is capable of multiplying selectively in tumors and inhibiting their growth. Several factors significantly influenced the tumor colonization of *Salmonella*. It was noticed that different administration routes could affect *Salmonella* colonization. Intraperitoneal injection of *Salmonella* resulted in less tumor colonization compared to intravenous injection [1]. Previously, we also found that *Salmonella* could target the untreated tumor when injected intratumorally into one of the bilaterally implanted tumors. These results indicated that *Salmonella*, administered via either intratumoral or systemic route, were able to accumulate in the tumors at distant sites [2]. In addition, oral administration of *Salmonella* still had antitumor activity and reduced toxicity [3]. *Salmonella* has been developed as oral vaccine vector for a number of infectious disease and several types of cancer [4]. To develop oral administration route for tumor-targeting *Salmonella*, we tested whether *Salmonella* could be orally as a therapeutic antitumor agent. The results suggest that oral administration of *Salmonella* not only colonized within tumors, but also led to significant antitumor immunoresponses. In this study, we want to elucidate the potential mechanism of antitumor effects by oral delivery *Salmonella*.

Material and methods

Cells, bacteria and mice

Mouse colorectal cancer (CT26) was cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 50 μg/ml gentamicin, 2 mM L-glutamine, and 10% heat-inactivated fetal bovine serum (FBS) at 37°C in 5% CO_2. A vaccine strain of S. *Choleraesuis* (ATCC 15480) was obtained from Bioresources Collection and Research Center (Hsinchu, Taiwan). This rough variant of S. *Choleraesuis*, designated vaccine 51, was obtained by spreading an 18-h broth

culture of the virulent strain 188 of S. *Choleraesuis* strain Dublin over the surface of a dried nutrient agar plate and placing a drop of a suspension of salmonella anti-O phage no. 1, and selecting for a phage-resistant colony after incubation at 37°C for 24 h [5]. Male BABL/c mice at the age of 6 to 8 weeks were obtained from National Laboratory Animal Center of Taiwan. The animals were maintained in a specific pathogen-free animal care facility under isothermal conditions with regular photoperiods. The experimental protocol adhered to the rules of the Animal Protection Act of Taiwan, and was approved by the Laboratory Animal Care and Use Committee of the China Medical University.

Animal studies

The mice were inoculated subcutaneously (s.c.) with 10^6 tumor cells. When the tumors had grown to 50 mm^3 to 100 mm^3, the mice were oral administered with *Salmonella* (2×10^6 colony-forming units; cfu) at day 7 for continuous 5 days. At various time points post infection, groups of mice were sacrificed, and the numbers of *Salmonella* in the tumors, livers, and spleens were determined on LB agar plates and expressed as cfu per gram of tissues. In a separate experiment, palpable tumors were measured every 5 days in two perpendicular axes with a tissue caliper and the tumor volume was calculated as: (length of tumor) × (width of tumor)^2 × 0.45, and body weight, the survival of the mice in the treated and control groups was monitored daily.

Assessment of cytokines and immunofluorescence staining

To determine the expression of inflammation cytokines (tumor necrosis factor-α, TNF-α and interleukin-1β, IL-1β) after oral administration *Salmonella*, mice were inoculated with CT26 cells (10^6) at day 0. Then, the groups of mice were treated with *Salmonella* (2×10^6 cfu) by oral administration at day 7 for continuous 5 days. To detect the protein and cytokine expressions, the organs were collected at day 16. Levels of inflammation cytokines in the tissue homogenates or sera were determined by ELISA (R & D, Minneapolis, MN). The protein content in each sample was determined by bicinchoninic acid (BCA) protein assay (Pierce Biotechnology, Rockford, IL). To analyze cell infiltrates in the tumors, groups of 4 mice that had been inoculated s.c. with 10^6 CT26 cells at day 0 were oral delivery with 2×10^6 cfu of *Salmonella* at day 7 for continuous 5 days. Control mice received PBS. The tumors were excised and snap frozen at time point. Cryostat sections (5 μm) were also prepared, fixed, and incubated with rat anti-mouse Ly-6G (Gr-1) (RB6-8C5, BD Biosciences, San Diego, CA), rat anti-mouse CD4 (L3T4) (GK1.5, BD Biosciences), or rat anti-mouse CD8a (Ly-2) (53-6.7, BD Biosciences) antibody. After sequential incubation with appropriate fluorescein isothiocyanate (FITC)-labeled secondary antibody, the slides were counterstained with 4',6-diamidino-2-phenylindole (DAPI). The infiltrating cells were quantified by averaging the number of each cell type in three areas of highest cell density at × 400 magnification in each section [6]. Terminal deoxynucleotidyltransferase-mediated deoxyuridine triphosphate nick end-labeling (TUNEL) assay was used to detect cell apoptosis within tumors and was performed according to the manufacturer's instructions (Promega, Madison, WI). Nuclei were stained with 50 μg/ml of DAPI. TUNEL-positive cells were counted under the microscope. We counted three high-power (× 400) fields that showed highest density of positive-stained cells per field to determine the average percentage of apoptotic (TUNEL positive) cells in each section [7].

Immunoblot analysis

The protein content in each sample was determined by BCA protein assay (Pierce Biotechnology). Proteins were fractionated on SDS-PAGE, transferred onto Hybond enhanced chemiluminescence nitrocellulose membranes (Amersham, Little Chalfont, UK), and probed with Indoleamine-pyrrole 2,3-dioxygenase (IDO) (Thermo Scientific, Rockford, IL) or monoclonal antibodies against β-actin (AC-15, Sigma Aldrich). Horseradish peroxidase-conjugated goat anti-mouse IgG or anti-rabbit IgG (Jackson, West Grove, PA) was used as the secondary antibody and protein-antibody complexes were visualized by enhanced chemiluminescence system (Amersham) [8-10].

Statistical analysis

The unpaired, two-tailed Student's t test was used to determine differences between groups for the comparisons of body weight, tumor volume, cytokine expression, the numbers of

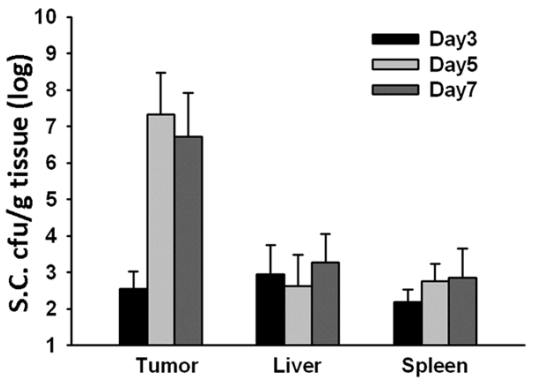

Figure 1. Preferential accumulation of *Salmonella* (S.C.) in the tumor microenvironment of mice administered orally with *Salmonella*. Mice bearing CT26 tumors were oral administered with 2×10^6 cfu of *Salmonella* for continuous 5 days. The amounts of accumulated *Salmonella* in tumors, livers, and spleens were determined on 3, 5, and 7 day post infection (p.i.). Each value represents mean ± SD from 4 mice.

$CD4^+$, $CD8^+$ and neutrophils and the number of apoptotic cells. The survival analysis was performed using the Kaplan-Meier survival curve and log-rank test. Any *P* value less than 0.05 is regarded statistically significant.

Results

Preferential accumulation in tumors via oral administration Salmonella

To determine the localization of oral administration *Salmonella*, we treated mice bearing syngeneic tumors with *Salmonell*a, and monitored bacterial burdens in the tumors, livers, spleens, and blood at various time points. As shown in **Figure 1**, five days after *Salmonella* inoculation, the amount of *Salmonella* (cfu/g tissue) in the tumors was approximately four to five orders of magnitude higher than that found in the livers or spleens in tumor-bearing mice. Even at day 7, *Salmonella* could still be detected in the tumor. Collectively, these results demonstrate that *Salmonella*, when oral administration to mice bearing established tumors, preferentially accumulated and retained in large amounts in the tumors for at least one week.

Effects of oral administration Salmonella on cytokine induction in mice

Because significant proinflammatory cytokine induction occurs with *Salmonella* infection, the

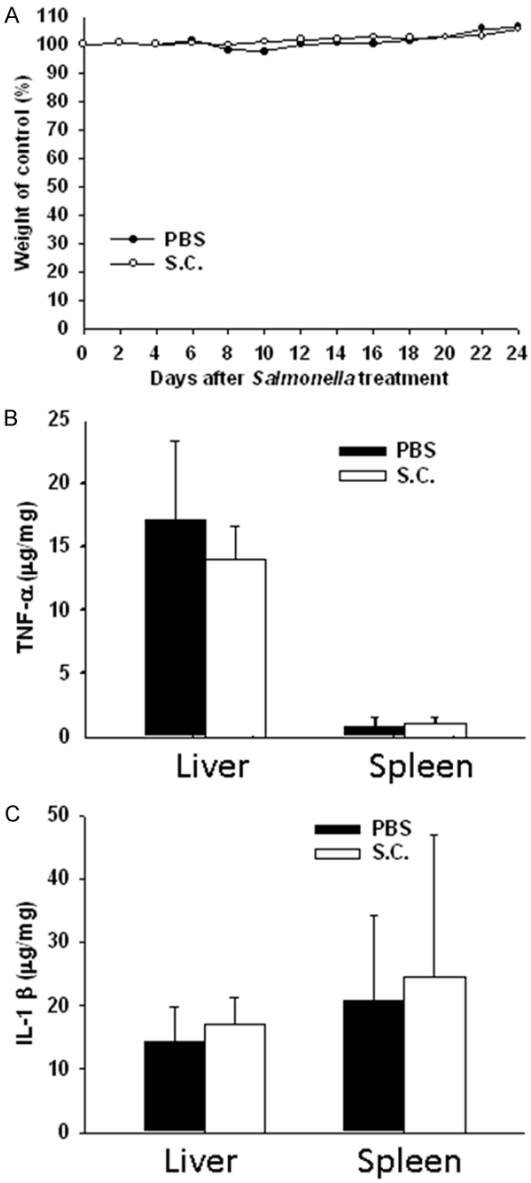

Figure 2. Susceptibility of mice to infection with *Salmonella* (S.C.). Mice were injected orally with *Salmonella* (2×10^6 cfu) for continuous 5 days; (A) The body weights and (B) TNF-α and (C) IL-1β levels were determined. The data are reported as means ± SD (n = 4).

administration of agents such as antibiotics may reduce the biological activity of these cytokines and their side effects. To examine the host inflammation response induced by *Salmonella*, tumor-bearing mice were orally treated with *Salmonella*, and body weight was measured. As indicated in **Figure 2A**, the body weights of mice treated with *Salmonella* were not significantly decreased compared with control group. After oral *Salmonella* treatment, the levels of inflammatory cytokines including

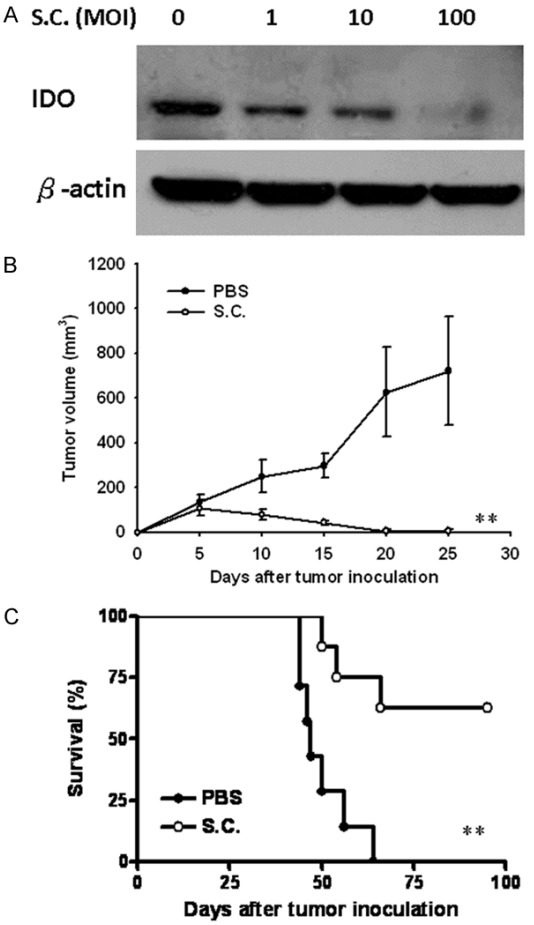

Figure 3. Antitumor effects of *Salmonella* on mice bearing CT26 tumors. (A) *Salmonella* reduced IDO protein expression in CT26 cells in a dose-dependent manner. After exposure to *Salmonella* (MOI: 0-100) for 4 h, the expression of IDO levels in CT26 cells were determined by immunoblot analysis. Groups of 7-8 BALB/c mice that had been inoculated subcutaneously with CT26 cells (10^6) on day 0, were injected orally with 2×10^6 cfu of *Salmonella* on day 7 for continuous 5 days. Control mice only received PBS. Tumor volumes (mean ± SEM, n = 7-8) among different groups were compared on mice bearing (B) CT26 tumors. Kaplan-Meier survival curves of mice bearing (C) CT26 tumors with different treatments are shown. Data were analyzed by the log-rank test. **, $P < 0.01$.

TNF-α (**Figure 2B**) and IL-1β (**Figure 2C**) were measured in the livers, spleen. Regardless of whether the mice were naïve or immunized, the induction of inflammation cytokines (i.e., IL-1β and TNF-α) in mice treated with *Salmonella* was not significantly different compared with the induction by PBS treatment (**Figure 2B** and **2C**). Taken together, these results suggest that oral administration *Salmonella* had a greater tumor-targeting efficiency with potentially lower side effects in the host.

Inhibition of tumor growth by oral administration Salmonella

We hypothesize that *Salmonella* breaks tumor immune tolerance via suppressing IDO as a crucial mediator of tumor-mediated immune tolerance by causing T cell suppression via tryptophan starvation in a tumor environment. Furthermore, to examine the effect of *Salmonella* on IDO levels in CT26 cells, CT26 cells were incubated with different multiplicity of infection (MOI) of *Salmonella*, and then analyzed by immunoblot analyses. Treatment of CT26 cells with MOI 0, 1, 10 or 100 of *Salmonella* caused a dose-dependent decrease in IDO levels compared to controls (**Figure 3A**). Antitumor effects of *Salmonella* were evaluated in terms of tumor growth and survival in mice bearing CT26 tumors. **Figure 3B** shows that tumor growth was significantly retarded in mice treated with *Salmonella* compared with that in PBS-treated control mice. The mean tumor volume in *Salmonella*-treated group was lowered by 98.60% compared with that in PBS-treated groups. **Figure 3C** shows that survival of the mice treated with *Salmonella* was significantly prolonged compared with that treated with PBS. *Salmonella* completely inhibited tumor growth in the four mice of *Salmonella*-treated group. The host memory immunresponses were observed by challenging high dose tumor cells (5×10^6) and the mice did not develop the tumor. Taken together, *Salmonella* exerted antitumor effects on CT26 tumor models. *Salmonella* accumulated in tumor microenvironment and induced strong host immune cells infiltrating into tumor. Infiltrates of T-cells and neutrophils within tumors from CT26-tumor-bearing mice inoculated with *Salmonella* were analyzed by immunohistochemistry. The results of immunohistochemical staining are shown in **Figure 4A**. A notable increase of neutrophils and T-cells infiltrates in the tumors was observed in *Salmonella* -treated mice. As shown in **Figure 4B**, the number of neutrophils and T-cells infiltrates in *Salmonella* -treated mice was obviously increased compared with that in the control groups. We then measured the numbers of apoptotic cells in TUNEL (**Figure 4A**). The number of apoptotic cells in the *Salmonella*-treated group was significantly

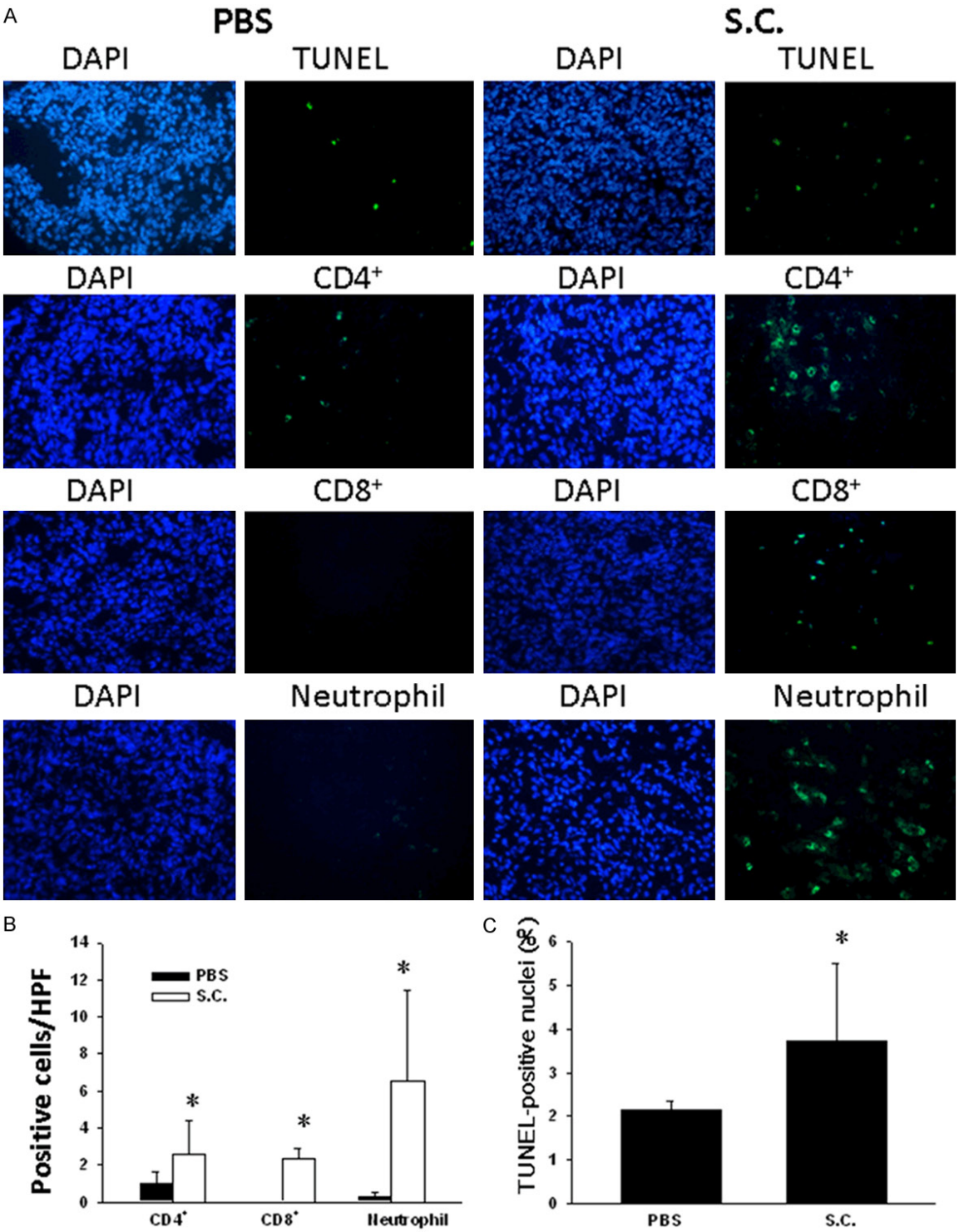

Figure 4. Increase of cellular infiltrates and apoptosis in the tumors from mice treated with *Salmonella*. Groups of 4 BALB/c mice that had been inoculated subcutaneously with CT26 cells (10^6) on day 0, were injected orally with 2×10^6 cfu of *Salmonella* on day 7 for continuous 5 days. Control mice only received PBS. A. Tumors were excised on day 16 and immunostained with TUNEL assay to analyze apoptotic cell (× 400) and with antibodies against Gr-1, $CD4^+$ or $CD8^+$ (× 400). B. Neutrophils $CD4^+$ and $CD8^+$ T-cell infiltrates were determined by averaging the cell numbers from three independent fields at × 400 magnification in each section (mean ± SEM n = 4). C. TUNEL-positive cells (mean ± SEM n = 4) were counted from three random fields in each section to determine the apoptotic index. *, $P < 0.05$.

higher than that in the PBS-treated (**Figure 4C**). Taken together, these results reveal that oral administration *Salmonella* was capable of inhibiting tumor growth, enhancing cellular infiltration into tumor regions and increasing the death of tumor cells.

Discussion

In the experiments described above, we prove that *Salmonella* is capable of targeting and multiplying in primary tumors via oral administration. Using murine colorectal tumor models, we demonstrated that orally administered *Salmonella* preferentially accumulated and amplified within implanted tumors in mice, achieving the ratio of the tumor to normal tissue 1000-10000 to 1. *Salmonella* preferentially targets tumors, with numbers far exceeding those in the liver, which is a normal site for *Salmonella* replication in non-tumor-bearing mice. The propensity of *Salmonella* to target primary tumors may also contribute to enhance host immunoresponses, as well as prolonging the survival of tumor-bearing mice. Our results also suggested that oral delivery *Salmonella* induced a strong additive effect on delaying tumor growth and enhancing survival of the mice.

Salmonella can induce multiple signaling pathways in tumors [11]. *Salmonella* has been widely studies; especially with respect to its immunopotentiating properties its ability to enhance host immunoresponse [12, 13]. However, the mechanism by which *Salmonella* inhibited tumor and the issue of whether that mechanism involved the reversal of IDO is not completely clear. IDO is an inducible enzyme that catalyzes the rate-limiting first step in tryptophan catabolism. IDO causes immunosuppression through breakdown of tryptophan in the tumor microenvironment. The depletion of tryptophan and toxic catabolites renders effector T cells inactive and dendritic cells immunosuppressive [14]. The present studies described an investigation of the effects of *Salmonella* on downregulation of IDO expression. The successful induction of immunity against poorly immunogenic malignancies is a major challenge for cancer therapy. Previously, we have demonstrated that host immune responses cooperate with *Salmonella*-mediated tumor destruction [2, 11]. Herein, we show that oral delivery of *Salmonella* results in substantial control of CT26 tumors. The reduction of IDO expression with tumor colonization by *Salmonella* results in the intratumoral recruitment of neutrophil and T cells, which may promote the induction of apoptosis of tumor cells. Strong neutrophils activation can cause tissue damage and this represents the basis for tumor destruction [15].

Salmonella is a powerful antitumor agent due to its tumor-targeting potential, antitumor capability, and ability to deliver therapeutic gene [16]. Host factors including innate and adaptive immune responses play roles in *Salmonella*-induced antitumor activity. Oral delivery is a relatively novel method for *Salmonella* cancer therapy. Previously, mice treated with *Salmonella* had a 9% lower average body weight compared with naïve mice treated with PBS during systemic injection [17]. In contrast, the body weights of mice treated with oral delivery-*Salmonella* were not significantly decreased. It was also reported that oral administration may provide an alternative route for low toxic delivery of *Salmonella* for effective antitumor therapy [18].

In summary, oral delivery-*Salmonella* displayed lower toxicity and improved efficacy and safety. Oral-delivery *Salmonella* also can provide a useful platform for oral, gastric or colorectal tumor, perhaps allowing other chemotherapeutic drugs to combine with tumor-targeting *Salmonella*. By taking advantage of the tumor-targeting activity and the stimulating host immunity activities of *Salmonella*, this system appears to hold promise for tumor treatment. These results yield insight into the complex interactions between *Salmonella* and host immunity, maximizing the possibility of therapeutic success. Therefore, oral administration *Salmonella* has promising potential for further clinical studies.

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Disclosure of conflict of interest

None.

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