

Obligate anaerobic *Salmonella typhimurium* strain YB1 treatment on xenograft tumor in immunocompetent mouse model

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Abstract. The present authors have previously reported a novel approach to genetically engineer *Salmonella typhimurium* for the medically important therapeutic strategy of using bacterial agents to target malignant tumors in a breast cancer tumor-bearing nude mouse model. However, studying an immunocompromised mouse model for cancer therapy is insufficient, as certain crucial information about the influence of the immune system may be missing. In the present study, inoculation of the *Salmonella* strain, YB1, into a colon cancer tumor-bearing immunocompetent mouse model was investigated. The present study determined the tumor targeting efficiency, antitumor potential, the effects of multiple treatments and the systemic toxicity. Intravenous inoculation of YB1 in BALB/c mice exhibited high antitumor effects and also greatly increased the tumor targeting ability and safety compared with the previously-reported nude mouse model. In addition, repeated administration of YB1 further enhanced this effect. Furthermore, no marked toxicity was observed with YB1 treatment, while the VNP20009 and SL7207 strains demonstrated certain adverse effects. The findings of the present study indicate that the YB1 strain is effective and safe in targeting a colon cancer tumor in an immunocompetent mouse model.

Introduction

The potential use of certain obligate and facultative anaerobic bacteria in cancer treatment has been investigated for numerous years (1). Bacteria, including *Bifidobacterium*, *Clostridium* and *Salmonella*, have been demonstrated to preferentially target and replicate in the hypoxic and necrotic regions of tumors, resulting in tumor repression (2-9). In our previous study, in order to strengthen the tumor hypoxia targeting efficiency and antitumor effect, a synthetic biology approach was used to generate a novel *Salmonella* strain, YB1 (10). The principle of YB1 is to regulate an essential gene (*asd*) of *Salmonella* using a hypoxia-induced promoter (10). Since *Salmonella* is sensitive to the expression of the *asd* gene, any leaky expression will destroy the entire regulation. Therefore, a genetic circuit was designed to neutralize the leakage, which was controlled by an anaerobic promoter in order to regulate forward transcription of *asd*, and another aerobic promoter was constructed to generate anti-sense *asd* mRNA (10). With this combination, YB1 only survived in an atmosphere with <0.5% O₂, which is equivalent to the anaerobic conditions inside solid tumor tissue (11), and the growth of YB1 was inhibited when exposed to increased oxygen levels that are equivalent to those in a normal organ (2-9% O₂) without additional diaminopimelic acid (DAP) (10,12). In the breast tumor-bearing nude mouse model, YB1 specifically colonized and proliferated in hypoxic/necrotic areas of the tumor, and retarded tumor growth (10).

In the present study, the antitumor effects of the YB1 strain were further investigated. The tumor targeting efficiency and safety evaluation were investigated in a solid tumor model using the colon cancer cell line, CT26. In addition, inoculation of YB1 in an immunocompetent mouse model (BALB/c mice) was compared with the previously-reported immunocompromised mouse model (nude mice). Furthermore, multiple YB1 injection treatments were assessed and the treatment of different tumor sizes was investigated. The present study, using the genetically engineered YB1 strain, provides preclinical data on the efficiency and safety of *Salmonella* mediated cancer therapy, which may further facilitate its optimization to improve the anticancer effect and reduce adverse effects in future clinical trials.

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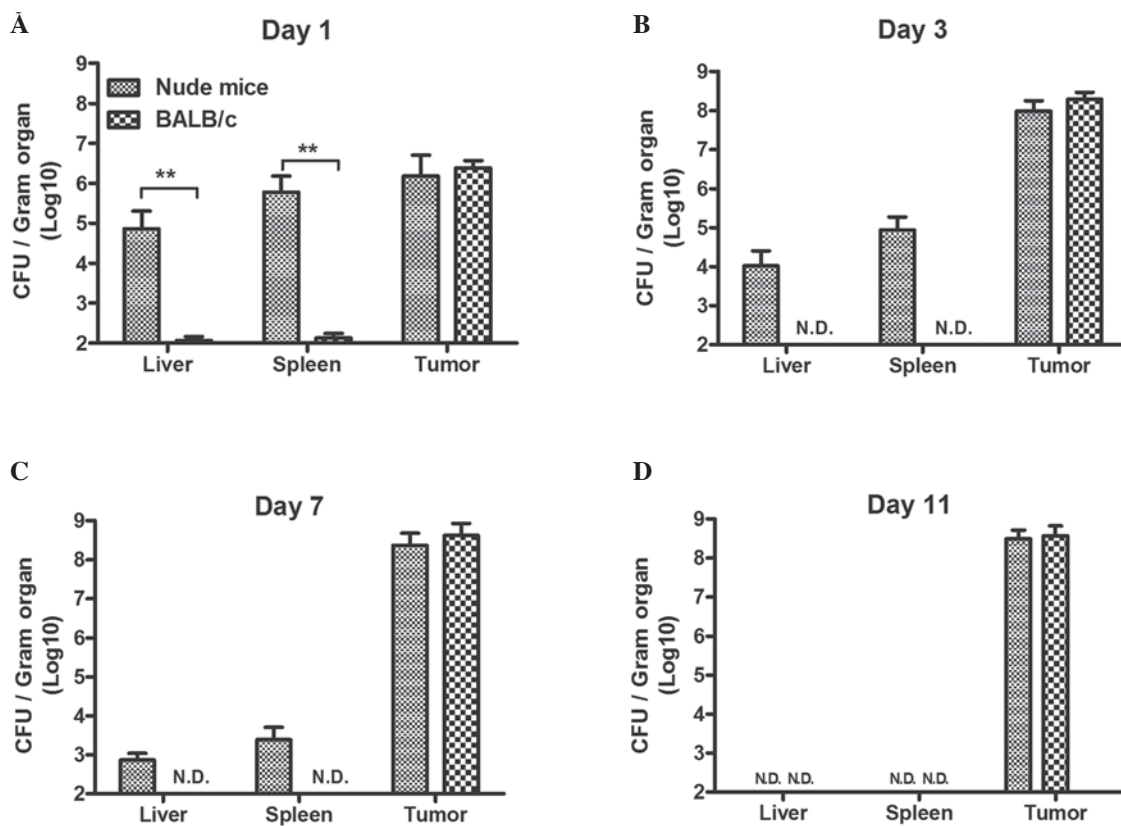


Figure 1. CFU test of YB1 in colon tumor-bearing nude and BALB/c mice. Nude and BALB/c mice with CT26 tumor received tail vein intravenous injections of YB1. Mice were euthanized at various time points, including days (A) 1, (B) 3, (C) 7 and (D) 11. The liver, spleen and tumor were collected and homogenized. CFU counts per gram of tissue are presented as the mean \pm standard deviation ($n=6$ mice). ** $P<0.01$. CFU, colony-forming unit; N.D., not detected.

Materials and methods

Bacteria, cell lines and animals. The *Salmonella typhimurium* strain, SL7207, was obtained from lab stock (13). The VNP20009 strain was purchased from the American Type Culture Collection (#202165; Manassas, VA, USA). The YB1 strain was constructed as described previously (10). Bacteria were cultured in lysogeny broth (LB) medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with chloramphenicol and DAP (Sigma-Aldrich) at 37°C. The CT26 colon carcinoma cell line was provided by Dr Songyue Zheng (University of Hong Kong, Hong Kong, China). Cells were cultured in high-glucose Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and 1% penicillin-streptomycin (Gibco Life Technologies, Carlsbad, CA, USA). Four-week-old female BALB/c nude mice (weight range, 16-20 g) and eight-week-old BALB/c mice (weight range, 18-20 g) were purchased from the Laboratory Animal Unit of The University of Hong Kong. The research protocols were approved and followed by the committee on the Use of Live Animals in Teaching and Research of the University of Hong Kong (CULATR no. 2689-12). Antibiotic and chemical working solutions were prepared as follows: Chloramphenicol, 25 $\mu\text{g}/\text{ml}$ in methanol; DAP, 50 $\mu\text{g}/\text{ml}$ in water.

Tumor-bearing mouse model. A total 5×10^5 CT26 cells were injected into the fat pad of the chest in nude mice or BALB/c mice. The tumor volumes were calculated using the following formula: $4/3 \pi (\text{height} \times \text{width}^2)/8$. When the tumors grew to ~ 500 or 100 mm^3 , the mice underwent bacterial treatment

and were grouped as follows: YB1-treated BALB/c group; YB1-treated nude group; VNP20009-treated BALB/c group; and SL7207-treated BALB/c group. Phosphate-buffered saline (PBS) treatment was used as a control. If the tumor size reached 20 mm in height or the volume was $\geq 4,000 \text{ mm}^3$, the mouse was euthanized (14). To measure YB1 distribution following treatment at different time points in nude mice and BALB/c mice, 5×10^7 colony-forming units (CFU) of YB1, VNP20009 or SL7207 were injected intravenously into CT26 tumor-bearing mice through the tail vein. The mice were sacrificed by intraperitoneal injection with pentobarbitone (University of Hong Kong) at different time points (day 1, 3, 7 or 11). The calculation of YB1 in tissues, survival rates and body weight analysis followed the methods previously described (10).

Tumor targeting potential and clearance of YB1. The tumor targeting potential and the clearance of YB1 in normal tissues were compared in immunocompetent (nude) and immunocompromised (BALB/c) mice. CT26 tumor cells were injected subcutaneously into nude and BALB/c mice. When the tumor volumes reached $\sim 500 \text{ mm}^3$, a single shot of YB1 was injected intravenously through the tail vein. At the indicated time points (day 1, 3, 7 or 11), the mice were euthanized by intraperitoneal injection with pentobarbitone and the liver, spleen and tumor were isolated, homogenized in LB and cultured on LB agar plates supplemented with antibiotics and DAP.

Multiple-shot YB1 treatment in BALB/c mice CT26 cancer model. A total of 1×10^5 CT26 tumor cells were injected subcu-

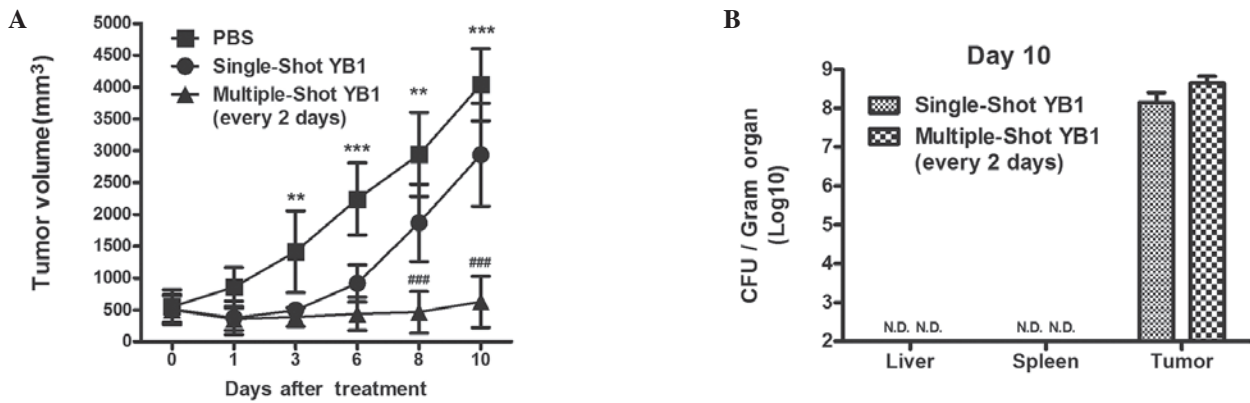


Figure 2. Repression of tumor growth by YB1 treatment. (A) When the tumor volume of CT26 tumor-bearing BALB/c mice reached 500 mm³, YB1 was injected once (single-shot group) or every 2 days for total of 4 times (multiple-shot group; n=5, mean ± standard deviation). (B) CFU counts indicated YB1 distribution in organs after 10 days of treatment. **P<0.01 and ***P<0.001, PBS group vs. YB1 groups; ###P<0.001, YB1 single-shot vs. multiple-shot groups. PBS, phosphate-buffered saline; CFU, colony-forming unit.

taneously into 10 BALB/c mice. When the tumor volumes reached ~500 mm³, mice in the 'single-shot YB1' group (n=5) were intravenously injected with a single-shot of YB1, whereas mice in the 'multiple-shot YB1' group (n=5) received an intravenous injection of YB1 every two days (days 0, 2, 4 and 6). The tumor sizes in the two groups were measured on days 0, 1, 3, 6, 8 and 10. On day 10, all mice were sacrificed by intraperitoneal injection with pentobarbitone. The liver, spleen and tumor were then isolated, homogenized in LB and cultured on LB agar plates supplemented with DAP to determine YB1 distribution.

Single-shot of different Salmonella strains in small CT26 tumor models. A total of 1x10⁵ CT26 tumor cells were injected subcutaneously into 20 BALB/c and 10 nude mice. When the tumor volumes reached ~100 mm³, a single-shot of YB1 (n=5), VNP20009 (n=5), SL7207 (n=5), or PBS (n=5) was injected intravenously into BALB/c mice, whereas nude mice were intravenously injected with a single-shot of YB1 (n=5) or PBS (n=5). The tumor sizes in all groups were measured on days 0, 1, 3, 6, 8, 10 and 12. On day 12, all mice were sacrificed by intraperitoneal injection with pentobarbitone. The liver, spleen and tumor were then isolated, homogenized in LB and cultured on LB agar plates supplemented with DAP to determine the distribution of YB1, VNP20009 and SL7207.

Statistical analysis. Student's t-test was used to analyze the significance of the results of the CFU test and tumor growth measurements. Analyses were performed using GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA). Data are expressed as the mean ± standard deviation and P<0.01 was considered to indicate a statistically significant difference.

Results

Accumulation of YB1 in CT26 tumors and normal tissues in nude and BALB/c mice. For the nude mice group, the results were similar to those observed in our previous study in a breast cancer tumor-bearing nude mouse model (10). Between 1x10⁵ and 1x10⁶ CFU/g of bacteria were observed in all the tested tissues on day 1 (Fig. 1A). After one day, the YB1

levels in the liver and spleen declined rapidly. In the tumor tissues, the YB1 levels increased until they reached a plateau of 1x10⁸ CFU/g at day 3, which remained stable until day 11 (Fig. 1B-D). The tumor to liver ratio was 9,000:1 on day 3 (Fig. 1B; P<0.05) and 300,000:1 on day 7 (Fig. 1C; P<0.05). By day 11, YB1 was completely eliminated from the liver and spleen (Fig. 1D, P<0.01). Notably, the YB1 levels reduced much more quickly in the BALB/c mice compared with the nude mice. Within 24 h after treatment with YB1, the YB1 levels reduced to ~1x10² CFU/g in the liver and spleen (Fig. 1A), while between days 3 and 11 no YB1 was detected in the normal tissues (Fig. 1B-D). YB1 levels in the tumor demonstrated a similar pattern in nude mice, which reached a plateau of 2-4x10⁸ CFU/g at day 3 that was maintained until day 11 (Fig. 1B-D).

Antitumor effect of multiple-shot YB1 treatment in BALB/c mice CT26 cancer model (tumor size ~500 mm³). Since a large amount of YB1 accumulated in the tumor, its antitumor effect was measured. Tumor growth (from a volume of ~500 mm³ at the time of bacterial inoculation) following a single-shot YB1 treatment in BALB/c mice was initially inhibited for 3 days and then delayed compared with the PBS-treated group from days 3-10 (P<0.01 on days 3 and 8; P<0.001 on days 6 and 10) (Fig. 2A). Since YB1 was cleared in normal tissues within one day (Fig. 1), YB1 treatment was increased in tumor-bearing BALB/c mice via intravenous injections every two days (on days 2, 4, 6 and 8). The tumor growth was significantly repressed (P<0.001 on days 8 and 10) and the volume of the tumors remained ~500 mm³ in the 10 subsequent days (Fig. 2A). On day 10 following YB1 treatment, the single-shot and multiple-shot groups of mice were euthanized by intraperitoneal injection with pentobarbitone, and the bacterial number in different organs was counted. The residual YB1 in the liver and spleen of the two groups was eliminated. In the tumor tissues, the YB1 levels in the multiple-shot group were increased compared with the single-shot group (P<0.05; Fig. 2B).

Antitumor effect with single-shot of different Salmonella strains in small CT26 tumor models (~100 mm³). To determine the antitumor ability of YB1 in small tumors (~100 mm³), a single-shot of YB1 was administered to

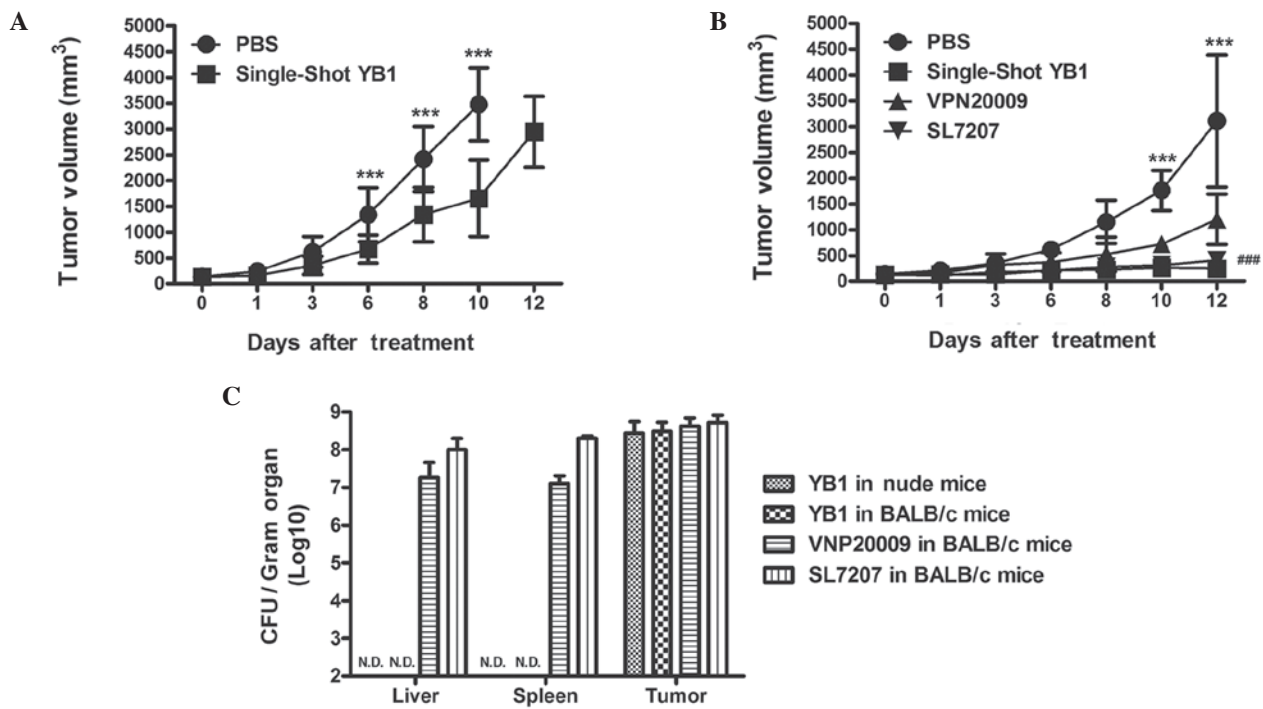


Figure 3. Repression of colon tumor growth by *Salmonella* strains in mice with a small tumor. CT26 colon tumor initial size $\sim 100 \text{ mm}^3$ in (A) nude mice or (B) BALB/c mice. Nude mice were treated with single-shot YB1 ($n=5$, mean \pm standard deviation). BALB/c mice were injected with PBS, YB1, VNP20009 or SL7207 ($n=5$ each; mean \pm standard deviation). (C) On day 12 after treatment, the mice of each group were euthanized and the bacteria in the organs were counted. $^{**}P<0.01$ and $^{***}P<0.001$, PBS vs. YB1, VNP20009 or SL7207 group; $^{###}P<0.001$, YB1 vs. VNP20009 group. PBS, phosphate-buffered saline.

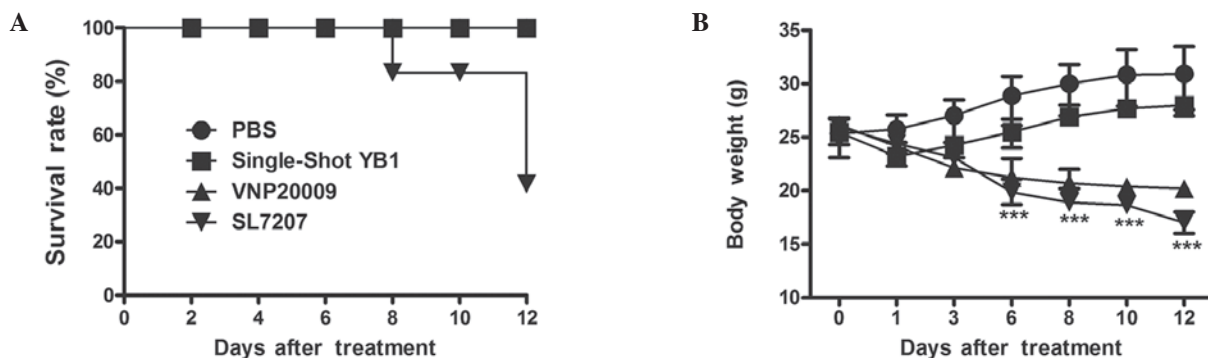


Figure 4. Evaluation of adverse effects of bacterial infection in CT26 colon tumor bearing BALB/c mice treated with PBS, YB1, VNP20009 or SL7207. (A) Survival rates and (B) body weights ($n=5$ each; mean \pm standard deviation) of the mice. $^{***}P<0.001$, PBS or YB1 group vs. VNP20009 or SL7207 treatment. PBS, phosphate buffered saline.

CT26 tumor-bearing nude and BALB/c mice (Fig. 3A and B). The results demonstrated that YB1 accumulated at approximately the same level in the two mouse models (Fig. 3C). The growth of the small tumors following inoculation with YB1 varied markedly, and tumor growth was delayed in nude mice but not in BALB/c mice (Fig. 3A). However, the overall growth of the tumor in BALB/c mice treated with a single-shot YB1 was significantly reduced compared with the PBS-treated mice, within the 12-day observation period (Fig. 3B).

The VNP20009 treatment repressed tumor growth in BALB/c mice for 6 days; however the tumor continued to grow after day 6 (Fig. 3B). The SL7207-treated mice demonstrated approximately the same pattern in tumor growth repression as YB1 inoculation in BALB/c mice (Fig. 3B).

However, although YB1, VNP20009 and SL7207 accumulated at similar levels in the tumor (2×10^8 CFU/g), significant differences were observed in the normal tissues (Fig. 3C). SL7207-inoculated mice demonstrated an uncontrolled infection as the bacterial level in the liver and spleen increased to 1×10^8 CFU/g (Fig. 3C). In VNP20009-treated mice, the bacterial levels were slightly reduced compared with the mice treated with SL7207, but were still $\sim 1 \times 10^7$ CFU/g in the liver and spleen (Fig. 3C).

Evaluation of the adverse effects of bacterial infection on weight gain and survival rate. Body weight and survival rate are major evaluation makers of treatment toxicity analysis for preclinical studies (15). Therefore, the adverse effects of the bacterial infection strategies used in the present study were

assessed based on the survival rate and body weight. Mice with small CT26 tumor models (~100 mm³) that were treated with single-shot YB1, VNP20009 or PBS survived during the 12-day observation period. However, a number of SL7207-treated mice succumbed on day 8, and the overall survival rate within the 12-day observation period was 40% (Fig. 4A). Following the first day of inoculation, all the treatment groups demonstrated significant body weight loss compared with the PBS-treated group (YB1, P<0.01; VNP20009 and SL7207, P<0.05); however, only the YB1-treated group started to recover after day 1 (P<0.01), whereas the other groups demonstrated continuous weight loss throughout the 12 days of observation (VNP20009, SL7207, P<0.001 on days 6-12). Although no mice succumbed in the VNP20009-treated group, their health condition was poorer than the YB1 group, due to the reduction in body weight following treatment (Fig. 4B).

Discussion

The physiology of solid tumors is different compared with normal tissues in certain aspects (16-20). Compared with normal organs, solid tumors consume a larger amount of oxygen and nutrients, a greater number of blood vessels supply the tissue and the vasculature within the tumor is highly abnormal with leaky vessel walls and reduced flow (16-18). Although tumors continuously generate new blood vessels, large regions of hypoxia are frequently observed in solid tumors (11,21,22). These regions of hypoxia lead to problems in cancer treatment and can result in resistance to radiotherapy or chemotherapy (21). However, the microenvironment of the solid tumor provides a haven for certain obligate or facultative anaerobic bacteria, such as *Salmonella* (23,24). When attenuated *Salmonella* strains are used as an anticancer vector, they are preferentially colonized in tumors with the ratio of titer being 1,000-10,000:1 in tumor over normal tissues. They may also evade monitoring by the immune system, and thus replicate and accumulate in the hypoxia region for long periods of time (1,7,8,25). Although *Salmonella* preferentially accumulates in tumor tissues, a proportion colonizes the normal tissues (10,26). In our previous study, recombinering technology was used to convert a *Salmonella typhimurium* strain into an 'obligate' anaerobe without otherwise interfering with the function of the bacterium. This avoids the problem of infection in normal aerobic tissues, as the modified strain lyses under these conditions. When in the hypoxic regions of a tumor, the bacterium thrives and functions as the wild type form as it is not compromised by an attenuation process. The efficacy of this approach was demonstrated by the effective tumor targeting and regression without damage to the normal organs in a situation where the unmodified parent strain is lethal to the host (10).

In our previous study (10), the animal model used to investigate *Salmonella* treatment included nude mice, which are deficient in T cell function. Mutations in the *Foxn1* gene in nude mice results in thymic aplasia and a lack of T cells, although the B cells remain unchanged (27). The immune system may aid bacteria to prevent cancer progression (28). A major obstacle for cancer immunotherapy is the ability of tumors to generate a microenvironment to evade the monitoring of the immune system. Although numerous methods have been developed to induce cancer immunity and certain interventions have demonstrated remarkably potent antitumor specific cytotoxic

responses (29-32), the majority of tumors did not regress following these treatments and continued to grow even when tumor specific T cells were introduced in the circulation (29,33). The reason for this may be due to the fact that their entry into the tumor masses was limited, or their functions were weakened and defected due to the downregulation of the specific target of antigen or major histocompatibility complex molecules inside the tumor (34). However, it has been demonstrated that, when combined with bacterial infection, the ability of the immune system to kill tumor cells was restored, which may aid in the goal to overcome immune escape mechanisms (28). Combined with *Salmonella* infection, tumor cells were destroyed by the immune system. In addition, it was demonstrated that tissue debris was captured and taken up by endogenous antigen-presenting cells, and presented on the cell surface of naïve T cells in order to generate tumor-specific T cells (35). Therefore, investigating the modified *Salmonella* strain in immunocompetent mice is an important experiment in moving towards clinical trials.

In the present study, an immunocompetent mouse model (BALB/c mice) was used, in which the clearance speed of YB1 in normal tissues was much faster compared with that in nude mice. While nude mice required at least 11 days to completely eliminate YB1, BALB/c required 1-2 days (Fig. 1). This may be due to a more efficient immune system in BALB/c mice compared with nude mice. In light of this improvement, the mice were treated with repeated injections. With high frequency boosting of YB1 every two days for a total of five treatments, the tumor growth was significantly repressed. These results indicated that the involvement of T cells did enhance the antitumor effects of YB1 treatment. Notably, when BALB/c mice with relatively small tumors (~100 mm³) were treated, a single injection was sufficient to prevent tumor progression (Fig. 3). Using the genetically engineered YB1 strain of *Salmonella* in cancer therapy demonstrated a promising antitumor effect, as well as high safety (Fig. 4). In conclusion, the approach of engineering *Salmonella* offers a feasible, effective and safe option for delivering bacteria in cancer therapy. In future studies, live animal imaging (intravital) technology may be applied to investigate the process of bacteria targeting tumors in more detail.

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