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Antitumor therapeutic effects of a genetically engineered *Salmonella typhimurium* harboring TNF-α in mice

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Abstract Although the use of TNF-α in the treatment of cancer is restricted due to its non-specific cytotoxicity and narrow range of applications to different cancers in clinical trials, we investigated a safe anti-cancer drug by the use of engineered bacterial capsule harboring TNF-α. The engineered bacterial capsule was designed to target cancer cells, promote a tumor-suppressive environment, and increase the efficacy of existing cancer treatments, including chemotherapy, radiotherapy, and cell therapy. The engineered bacterial capsule was constructed with *Salmonella* capsulizing TNF-α protein, which was produced and capsulized by *Salmonella* to reduce side effects of the protein. This bacterial capsule induced a tumor-suppressive environment through the activation of natural killer cells. Engineered bacterial capsule invaded tumor cells, released TNF-α, and induced apoptosis of tumor cells without apparent side effects. In a murine melanoma model, the bacterial capsule of TNF-α significantly inhibited tumor growth by 80–100% and prolonged the survival of the mice. When tested in combination with chemotherapy (cisplatin), antibiotics, and vaccine, recombinant microbial treatment increased the anti-tumor effects of existing therapies. The anti-tumor effects of the bacterial capsule of TNF-α were also observed in cervical cancer, melanoma, breast cancer, colon cancer, and renal carcinoma. These results suggest that the bacterial capsule of TNF-α is a promising strategy for TNF-α treatment.

Keywords Antitumor effects · Tumor treatments · TNF-α · *Salmonella typhimurium*

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Introduction

Tumor treatment currently focuses primarily on surgical excision, chemotherapy, radiation therapy, and immunotherapy (Jansen et al. 2009; Lissoni et al. 2009; Stupp et al. 2009). Although the effectiveness of these therapies has increased remarkably, their use is still restricted due to non-specific cell cytotoxicity, a narrow range of applications to different tumors, and the side effects of high-dose treatment (Le Pechoux et al. 2009).

In melanoma, surgical excision is the principal treatment for early-stage tumors. The use of chemotherapy and radiation therapy to treat melanoma is limited due to their side effects (Goren et al. 1986; Stewart et al. 1986; Stewart et al. 1988), but adjuvant therapies, such as interferon treatment, have been shown to promote survival in patients (Chen et al. 1992; Pajkos et al. 1998; Pisters and Evans 2008).

The goal of our work is to design tumor-specific treatments using TNF-α for chemotherapy, radiotherapy and immunotherapy. Toward this end, we have engineered a microbe that specifically targets various tumors, delivers drugs safely, and does not interfere with existing cancer therapies.

Salmonella, a facultative anaerobe, has been exploited as an antitumor agent that is capable of preferentially amplifying within tumors and inhibiting their growth (Saltzman et al. 1996; Bermudes et al. 2000; Zheng et al. 2000; Luo et al. 2001). In an effort to enhance therapeutic efficacy, this type of approach has been combined with gene-directed enzyme/prodrug therapy (King et al. 2002). For example, auxotrophic *Salmonella typhimurium* expressing prodrug-converting enzymes has been generated by transformation with a prokaryotic expression vector encoding herpes simplex virus thymidine kinase (Pawelek et al. 1997) or by chromosomal insertion of the *Escherichia coli* cytosine deaminase gene (Beck et al. 1972). *Salmonella* has also been exploited for the transfer of eukaryotic and prokaryotic expression vectors into mammalian cells in vitro and in vivo (Weiss 2003). Oral administration of attenuated *S. typhimurium* carrying an eukaryotic expression vector encoding interferon-gamma (IFN-γ) restores the production of this cytokine in the macrophages of IFN-γ-deficient mice (Paglia et al. 2000). When delivered orally to mice, *S. typhimurium* carrying eukaryotic expression vectors for cytokines (i.e., interleukin-12 or GM-CSF) mediates cytokine expression and exerts antitumor effects (Yuhua et al. 2001). Thus, it should be feasible to use *Salmonella* strains transformed with eukaryotic expression vectors to deliver various effector molecules to tumors, with the goal of enhancing antitumor activity.

The aim of our current work is to engineer *Salmonella* that produces tumor suppressor molecules without secreting into normal cells. We designed *Salmonella* to harbor a therapeutic protein, to be non-toxic to normal cells, and to be used to bombard tumors with suppressor proteins.

We focused on the suppressor protein TNF-α, since it is a major component of both innate and specific acquired immunity, and has the ability to induce apoptosis of tumor-associated cells, which can result in complete destruction of tumor cells (Green et al. 1979; Moriya et al. 1984; Ortaldo et al. 1986; Ziegler-Heitbrock et al. 1986; Lejeune et al. 2006).

However, studies in humans have shown that administration of high-dose recombinant cytokine is associated with serious side effects (Fichtner et al. 1990; Terlikowski 2002; Lejeune et al. 2006).

We postulated that local low-dose release of cytokine might overcome systemic toxicity without impairing immune recognition of malignant cells by the immune system (Terlikowski 2002). Because of its tumor-targeting and tumoricidal effects, we used *S. typhimurium* engineered to express TNF-α as a strategy for the effective administration of cytokine. A plasmid encoding the fusion protein SipB-TNF-α was used to create recombinant TNF-α-expressing *S. typhimurium*. SipB, a *Salmonella* invasion protein, induces apoptosis and localizes to the outer membrane during mammalian cell entry and in late exponential phase bacterial cultures (Hersh et al. 1999).

In the current study, we investigated the activity of *S. typhimurium* carrying a prokaryotic expression vector encoding TNF-α as a tumor-targeting anticancer agent and adjuvant to existing tumor therapies in syngeneic murine tumor models.

Material and method

Bacterial strains and growth conditions

We used the *S. typhimurium* BRD509 strain, which is an *aroA aroD* mutant of SL1344 (Hoiseth and Stoker 1981). Strains were grown on L-agar or in L-broth supplemented with 0.3 M sodium chloride. This strain was contributed by IS Lee (Hannam University, Korea).

Mouse

Balb/c and C57BL/6 mice were obtained from DaeHan BIOLINK (Korea). $CD8^{-/-}$C57Bl/6 (H-2^b) mice and $NK^{-}T^{+}$ C57Bl/6 transgenic mice lacking natural killer (NK) cells on C57BL/6 background were contributed by CW Hong, SH Park (Korea University, Korea). Mice were treated according to institutional animal care and use guidelines.

Tumor cell and challenge

We used various tumor cells for tumor model in mice. Tumors used are cervical tumor (TC-1), renal cell carcinoma (RENCA), colon cancer (CT-26), breast carcinoma (4T-1), EL4, and B16F10 cells. Syngeneic C57BL6 mice were injected subcutaneously with 10^4 tumor cells (TC-1, EL4, B16F10) in the thigh of 7-week-old female mice and BALB/c mice were injected (4T-1, CT-26, and RENCA). Tumor diameter was measured daily with a digital caliper. All animal experiments were performed in accordance with national animal care regulations.

Plasmid constructions

For the expression of the SipB 160 a.a-TNF-alpha fusion, pSSIM was used as vector. TNF-alpha fragment was amplified by primers (TNFL, 5′-agatctatgagcacagaaagcat-3′;

TNFR, 5′-ctcgagtcacagagcaatgactcc-3′) with Top10P (TNF-alpha encoding plasmid). pSSIM was digested by BglII and XhoI and ligased with this TNF-alpha fragment. This construct was named as pSST (Plac-sipB160-tnf alpha). Plasmid constructions in this study were transformed into *E. coli* DH5α. From the transformed DH5α, these plasmids were extracted and again transformed into *S. typhimurium* SF586. And the plasmids from the transformed SF586 were transformed again to *S. typhimurium* BRD509. All resulting protein fusions were checked by DNA sequencing.

Preparation of culture supernatant proteins and immunoblotting

Culture supernatant proteins were prepared as follows. In brief, 10 ml of bacterial supernatant were briefly passed briefly through a 0.45-μm pore-size syringe filter to remove bacteria, and was saved for analysis of secretory proteins. The cell pellets were resuspended in phosphate buffered saline (PBS) buffer (pH 8.6). Culture supernatant and pellets were separated in a 12% SDS-polyacrylamide gel and transferred to nitrocellulose membranes. SipB/TNF-alpha chimeric proteins were detected by immunoblot analysis. Western blots were treated with a polyclonal antibody against SipB, followed by incubation with a horseradish peroxidase-labeled anti-rabbit antibody. Blots were developed using a chemiluminescense detection kit.

Protocols for subcutaneous administration protocols

Bacteria were grown overnight until they reached mid-log phase. They were then harvested by centrifugation (3,000×*g*) and resuspended in a 10% sodium bicarbonate buffer. C57BL/6 mice were subcutaneously inoculated with the bacterial suspension (10^8 cfu/mouse) in a volume of approximate 100 μl. Each group of mice received the corresponding strain of recombinant *Salmonella*. Control mice received the buffer only.

Cytokine assay

Blood was collected from all mice before and after subcutaneous administration. The blood samples were stored at 5 °C for 12 h. After coagulation, sera were collected by centrifugation (5 min, 2,000×*g* at 4 °C). At the end of the experiment, samples of the same group that had been collected at the same time were pooled and tested by TNF-α sandwich ELISA kits (Bender Medsystems Inc., USA) with triplicate wells. Cytokine serum levels were measured by a powerful multiplexed assay combined with flow cytometry using commercially available kits BD™ Mouse TH1/TH2 Cytokine Cytometric Bead Array (CBA) according to the kit procedure (BD Biosciences Immunocytometry Systems and BD Bioscience Pharmigen, USA).

Histological analysis

Each mouse tissue were collected and sequentially immersion-fixed in 10% paraformaldehyde/0.1 M phosphate buffer (PB) pH 7.4, 12.5% glucose/0.1 MPB pH 7.4, and 25% glucose/0.1 MPB pH 7.4 for each 2 days. After the toes were decalcified using 5% ethylenediaminetetraacetic acid/25% glucose/0.1 MPB pH 7.4 for 2 days, they were routinely processed and embedded in 4% carboxymethyl cellulose. Standard sagittal sections measuring 5 μm in thickness were prepared from the frozen and fixed toe using a cryostat CM3050S (LIECA, Tokyo, Japan). The inflammation was histologically assessed on the sections, which were stained with Mayer's hematoxylin and eosin, toluidine blue pH 7.0, and tartrate-resistant acid phosphatase.

Invasion assay

Murine melanoma cells were infected with *Salmonella* strains, at an MOI of 100, for 1 h. After infection, cells were washed three times with PBS and then treated with 100 μg/mL of gentamicin (Invitrogen Corporation), for 1 h at 37 °C. After antibiotic treatment, cells were washed again with PBS and then incubated with 1% Triton X-100 for 5 min at 37 °C. The number of internalized bacteria was determined by plating tenfold serial dilutions of the cell lysates on LB plates. Invasion rate (%) were determined using the formula: $\text{Invasion rate } (\%) = \text{number of internalized recombinant bacteria} / \text{number of internalized wild} - \text{type salmonella} \times 100$

Cytotoxicity assay and apoptosis assay

For the analysis of cytotoxicity, we cultured tumor cells with sample after 24–48 h, and collected the culture supernatants. The supernatant was analyzed with CytoTox 96 non-radioactive cytotoxicity assay (Promega, USA). In the initial phase of apoptosis, the caspases became activated and the FLICA bound to these activated caspases. Mouse melanoma cells were treated with different microbe for 24 h. After 24 h, the cells were stained with FLICA Apoptosis detection kit caspase assay (Immunochemistry Technologies, LLC) according to the manufacturer's instructions.

Tumor implantation and evaluation of antitumor effects

For tumor implantation, 6- to 8-week-old female, C57BL/6 mice were implanted subcutaneously on the mid-right side with 10^5 B16F10 cells in 100 μl PBS. Tumors were allowed

to grow for 7 days before treatment. Bacterial strains administered next to the tumor. Mice were examined daily until tumors became palpable, and then their diameters were measured every other day in two dimensions, with a microcaliper. The antitumor activity of treatments was evaluated by measuring tumor growth inhibition. Tumors were measured individually with a caliper. Tumor volumes were determined using the formula: tumor volume = length × width^2 × 0.52. Survival time was used as one of the criteria for tumor inhibition. Mice surviving over 50 days and tumor-free animals were denoted as 50-day survivors. All animal experiments were approved by the institutional animal care committee in Korea University.

Chemotherapy and tumor regrowth delay assay

Each of microbe-treated mice received a single dose of the cisplatin. The mice received the dose of cisplatin during the same time window. Control experiments consisted of the injection of microbe. After treatment, the tumor growth was determined daily by measuring tumor diameter

Direct immunization with *Salmonella*

C57BL/6 mice were immunized twice with microbe, by intraperintoneal injection on day 0 and by subcutaneously injection on day 7. On day 30, animals were challenged with 1×10^4 B16F10 cells in PBS, were challenged with microbe, and were then analyzed of tumor growth and survival. Animals were monitored for tumor growth every 2–3 days for 60 days.

Statistical analysis

Statistical significance of the experimental results was determined by the Student's *t* test. For all analyses, $p<0.05$ was accepted as a significant probability level.

Results

Construction of a SipB/TNF-α secretion plasmid and expression of recombinant protein

To generate *S. typhimurium* harboring TNF-α, we constructed a SipB/TNF-α secretion plasmid (Fig. 1a). An amplified DNA fragment encoding TNF-α was fused to sequences encoding SipB to generate pSSTM (Plac-sipB160 TNF-α). *E. coli* DH5αE were transformed with pSSTM, and then the plasmid was extracted and used to transform *S. typhimurium* SF586. Plasmid from transformed SF586 cells was used to transform *S. typhimurium*

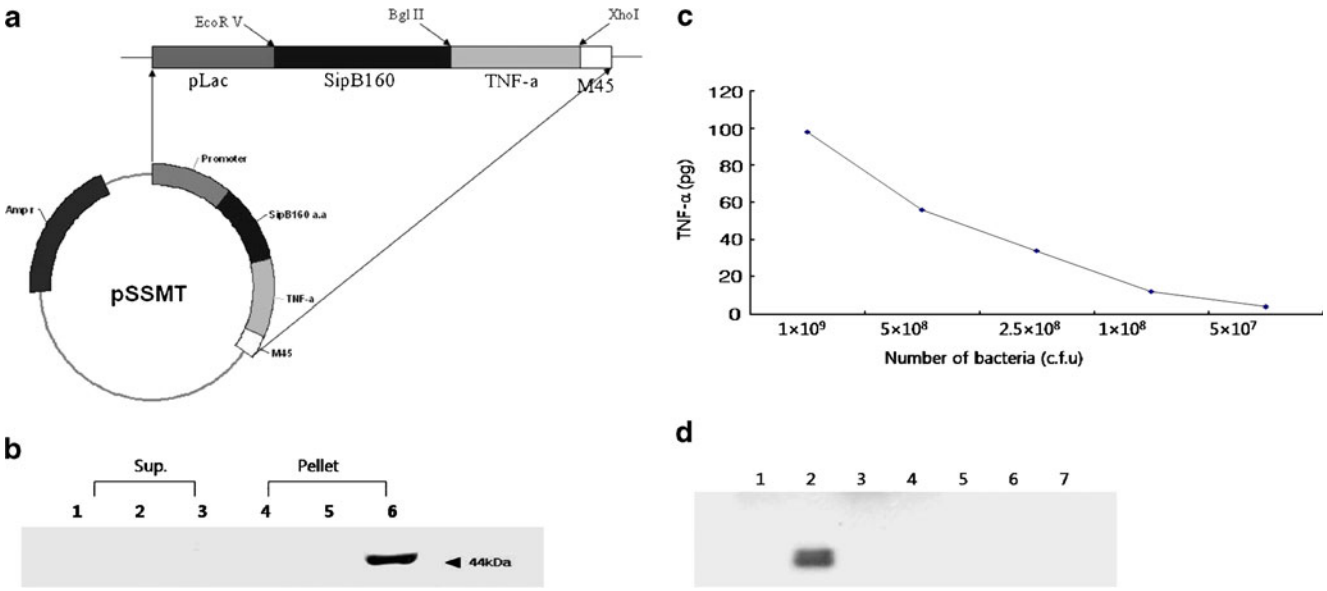

Fig. 1 Construction of recombinant *Salmonella*-harboring TNF-α. **a** Schematic presentation of plasmid harboring recombinant TNF-α protein. Carboxyl-terminal ends of these hybrid proteins were encoded by the designated plasmids. **b** The expression of recombinant TNF-α fusion proteins into culture supernatants by *S. typhimurium*. Recombinant TNF-α proteins were detected with a mAb to TNF-α. All recombinant TNF-α proteins were not secreted into the culture supernatant at comparable concentrations. Expression was analyzed by immunoblotting with whole bacterial lysates. *1* Supernatants of *S. typhimurium*, *2* supernatants of *S. typhimurium* with secretion plasmid vector alone, *3* supernatants of *S. typhimurium* with TNF-α secretion plasmid, *4* pellets of *S. typhimurium*, *5* pellets of *S. typhimurium* with secretion plasmid vector alone, *6* pellets of *S. typhimurium* with TNF-α secretion plasmid. **c** Quantification of harbored TNF-α proteins of bacteria. Bacterial lyates were examined with TNF-α specific ELISA. **d** *S. typhimurium*-harboring TNF-α were cultivated without antibiotics. Bacteria were collected daily, each bacterial lysates were immunobloted by anti-TNF-α protein antibody. *1* PBS, *2* 24 h-cultivation, *3* 24–48 h-cultivation, *4* 48–64 h-cultivation, *5* 64–88 h-cultivation, *6* 112–136 h-cultivation, *7* 160–184 h-cultivation

BRD509. All resulting plasmid sequences were verified by DNA sequencing.

We examined the protein expression of TNF-α from engineered *S. typhimurium* by immunoblot analysis of cell lysates and culture supernatants. We detected proteins of the expected molecular mass (44 kDa) of recombinant TNF-α in the cell lysates of all transformants, but not in the supernatants of cells cultivated in LB (Fig. 1b). To identify harbored proteins of recombinant bacteria, we examined bacterial lysates with ELISA. The quantitative yield of recombinant protein by bacterial transformants was about 100 pg of recombinant protein per 10^9 cells. In addition, recombinant bacteria expressed TNF-α during 24 h (Fig. 1c) and the expression of protein were only observed in 24 h (Fig. 1d). And recombinant *Salmonella* were reduced about 90% in tumor-bearing mouse after 24 h, this result suggested that the loss of bacteria induced releasing of TNF-α in tumor region (Electronic supplementary Fig. S1).

Engineered *Salmonella* induce tumor cell lysis by caspase activation

To determine the cytotoxic effects of engineered *Salmonella*, we treated B16F10 melanoma cells with recombinant bacteria. Recombinant *Salmonella* invaded tumor cells at a 50% higher invasion rate than normal *Salmonella* (Fig. 2a), and were able to kill the tumor cells. The results suggested that recombinant *Salmonella*-harboring TNF-α specifically kill B16F10 melanoma cells as compared to nornal strains (Fig. 2b, c), and that the cytotoxic effects of recombinant

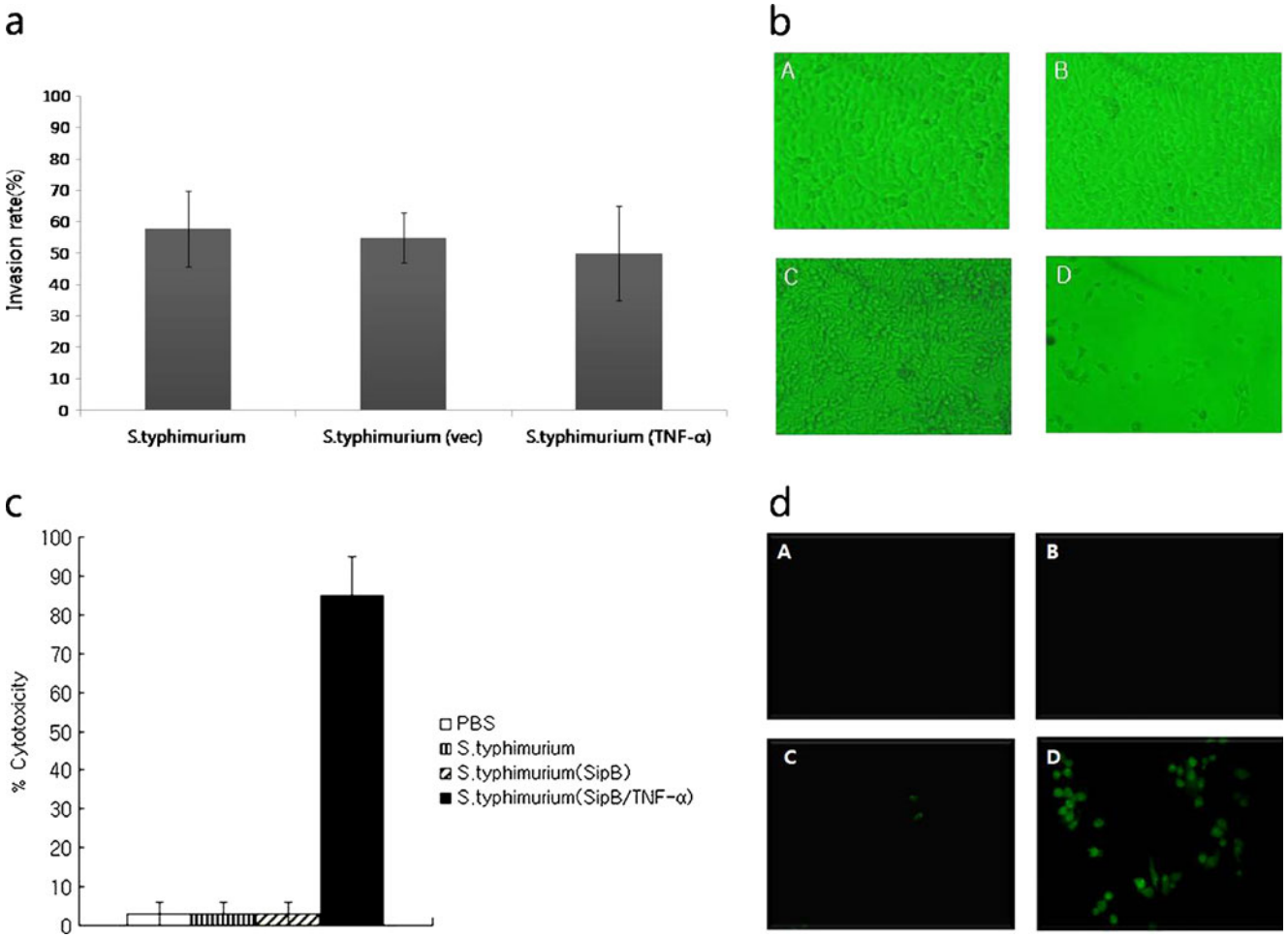

Fig. 2 Cytotoxicity of recombinant *Salmonella*-harboring TNF-α. **a** Bacterial invasion rates were examined. Bacterial invasion were tested in B16F10 melanoma cells. The relative percent value against normal *S. typhimurium* strains were calculated by Cytotox 96 cytotoxicity kit. **b** Suppression of tumor cell growth by the recombinant TNF-α proteins of *Salmonella*. Mouse melanoma B16.F10 cells were treated with PBS-A, and *S. typhimurium* alone-B, *S. typhimurium* with a control vector-C and *S. typhimurium* harboring TNF-α-D as shown in the methods section. **c** Direct melanoma cell killing activities of the recombinant TNF-α proteins of *Salmonella* in vitro. Mouse melanoma B16F10 cells were treated with PBS, and *S. typhimurium* alone, *S. typhimurium* with control vectors and *S. typhimurium* harboring TNF-α. **d** detection of apoptosis in tumor cells by the recombinant TNF-α proteins of *Salmonella*. Mouse melanoma B16.F10 cells were treated with PBS-A, and *S. typhimurium* alone-B, *S. typhimurium* with a control vector-C and *S. typhimurium* harboring TNF-α-D as shown in the FLICA apoptosis detection assay

bacteria on tumor cells are due to the induction of caspase activation (Fig. 2d)

Safety of *Salmonella*-harboring TNF-α in sera and tissue

We analyzed bacterial existence in mice after treatments for safety. In mice with subcutaneous inoculation of recombinant microbe, we did not detect microbe from spleen, liver, and blood (Fig. 3a). We analyzed TNF-α secretion in mice inoculated with engineered *S. typhimurium* harboring TNF-α. Blood was collected from mice before and after subcutaneous administration of recombinant bacteria. Samples from the same group were pooled, and TNF-α levels were analyzed by CBA method. The levels of TNF-α in C57BL/6 mice with *Salmonella*-harboring TNF-α were similar to those of mice inoculated with normal *S. typhimurium* (Fig. 3b). To verify the effect of recombinant bacteria on tissue, we examined histological changes in various tissues after administration of recombinant *Salmonella*. We found that recombinant *Salmonella* did not induce significant histological side effects, such as severe inflammations (Fig. 3c)

Engineered bacteria inhibit tumor cell growth and prolong survival

The tumor inhibitory activity of recombinant *Salmonella* was analyzed in C57BL/6 mice inoculated subcutaneously

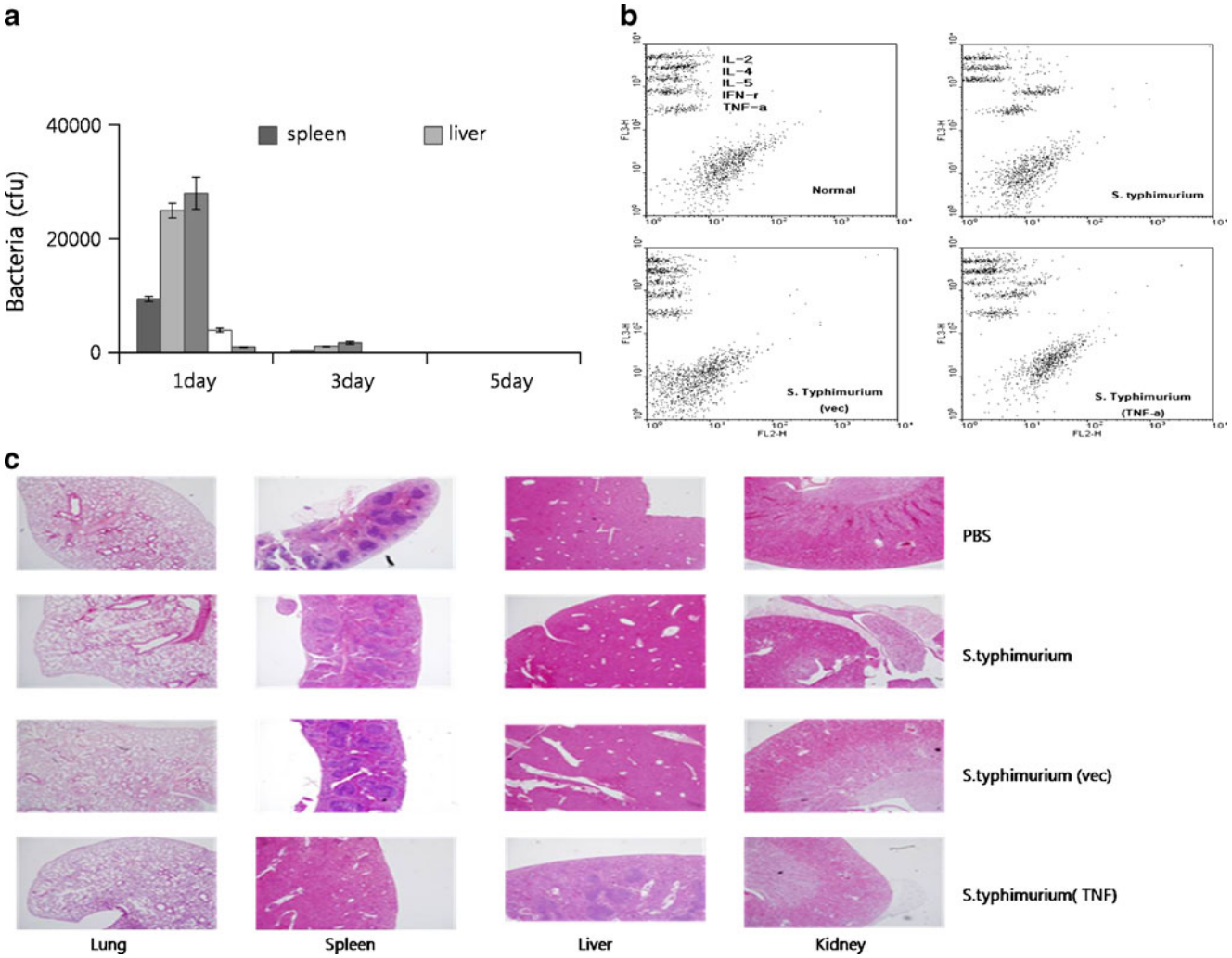

Fig. 3 Analysis of inflammation after subcutaneous-inoculation with recombinant bacteria. **a** Bacterial distribution was examined in mice after sc inoculations with 1×10^8 bacterial cells. At 5 days, bacteria were not cultivated from tissue in mice with subcutaneous recombinant salmonella inoculation. But in mice with wild-type *Salmonella* as positive control, bacteria were cultivated from tissue cultivated in medium. **b** Cytokine levels in sera of animals by delivery of *S. typhimurium* with and without vector by CBA assay. Serum was collected at 7 day after subcutaneous inoculations with 1×10^8 bacterial cells. **c** To examine severe inflammation by the recombinant TNF-α proteins of *Salmonella*. Histological sample were collected at 7 day after subcutaneous inoculations with 1×10^8 bacterial cells. Mice were treated with PBS, and *S. typhimurium* alone, *S. typhimurium* with a control vector (vec) and *S. typhimurium* harboring TNF-α. As shown in the H/E staining, all the samples were not shown severe inflammation

with B16F10 cells (10^4). Mice were inoculated at day 0; and at day 8, visible nodules had developed at all injection sites. Mice were inoculated subcutaneously with *Salmonella* on days 7 and 14, and were then examined daily until tumors became palpable, at which point, tumor diameter was measured in two dimensions with a micro-caliper every other day. Normal *Salmonella* and *Salmonella* carrying a control vector alone uniformly failed to elicit a protective response against lethal subcutaneous tumor cell challenge, and tumor growth in these mice was rapid and uniform. In contrast, there was a substantial decrease in tumor volume in mice treated with recombinant *Salmonella*-harboring TNF-α, with complete inhibition of tumor cell growth evident in all (90%) animals (Fig. 4a). Nine of ten mice that received *Salmonella*-harboring TNF-α exhibited a dramatic suppression of tumor growth and were cured of melanoma. In addition, mice with *Salmonella* and TNF-α recombinant protein were reduced tumor growth like mice with *Salmonella*-harboring TNF-α (data not shown).

We next assessed whether recombinant *Salmonella* prolonged survival time. Eight days after inoculation with 10^4 B16F10 cancer cells, all C57BL/6 mice developed tumors.

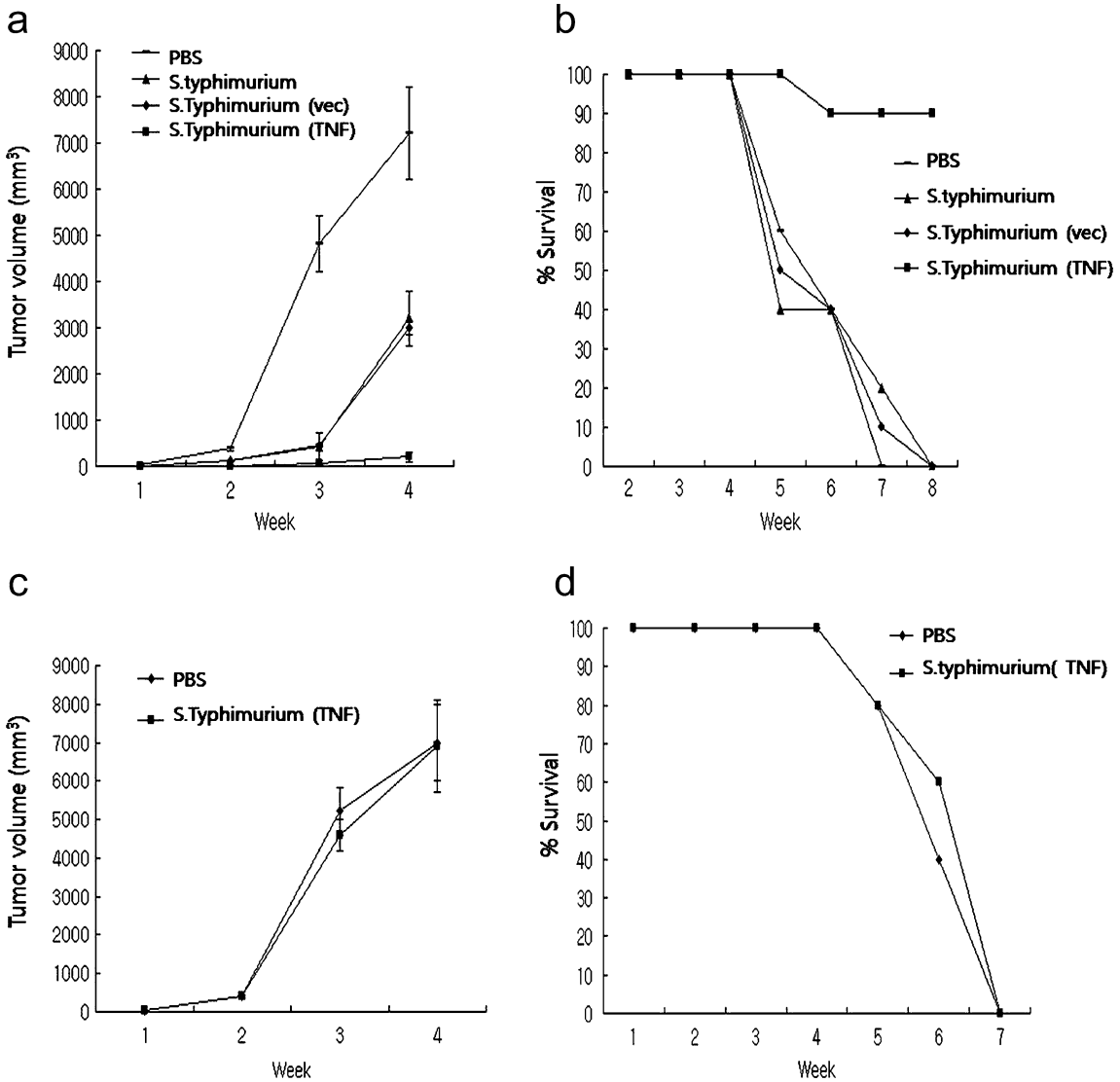

Fig. 4 Effect of *S. typhimurium*-harboring TNF-α in melanoma-bearing mouse. **a** Growth prevention of B16F10 tumors by a subcutaneous inoculation of *S. typhimurium* harboring TNF-α. Tumor-bearing mice were inoculated with *S. typhimurium* harboring TNF-α on day 7 and 14. Experimental animals in groups were each subcutaneously inoculated two times at 1 week intervals with 1×10^8 *S. typhimurium* alone, *S. typhimurium* with a control vector (vec) and *S. typhimurium*-harboring TNF-α. Animals were examined daily until the tumor became palpable, after which its diameter was measured with microcalipers in two dimensions every other day. **b** Survival was checked every day after inoculation. $^*P<0.05$ as compared with the control groups. To examine immunity against tumor challenge with *S. typhimurium* harboring TNF-α, cured mice were lethal challenged with 1×10^5 B16F10 melanoma cells. Tumor growth **c** and survival **d** were checked every day after inoculation. $^*P<0.05$ as compared with the control group (PBS)

Seven days after inoculation, mice were inoculated with *Salmonella*-harboring TNF-α, control vector, or no vector (blank control). The survival time of the TNF-α-treated group was much longer than that of the control vector and blank control groups (Fig. 4b). The cytokine-treated group tended to have a higher survival rate. To test whether the mice developed immunity against the tumor cells, eoght tumor-free mice received a second challenge (10^4 B16F10 cells). All of the formerly tumor-free mice developed tumors. Inhibition of tumor cell growth was not evident in any of the mice that received a second inoculation (data not shown). Furthermore, there was no significant difference in tumor growth inhibition (Fig. 4c) and survival between mice that received *Salmonella*-harboring TNF-α and the control groups following a second challenge (Fig. 4d). These results suggested that recombinant *Salmonella* alone does not elicit an immune response, but does induce direct protection against tumors.

Engineered *Salmonella* inhibit tumor cell growth by direct killing with TNF-α and by creating a tumor-suppressive environment

To investigate the mechanism of inhibition of tumor growth by engineered *Salmonella*, we implanted tumor cells and inoculated with recombinant *Salmonella* and TNF-α in mice. The result showed that combinatory effects with *Salmonella* and TNF-α were like those of recombinant *Salmonella*-harboring TNF-α (Fig. 5a). In further study of mechanism, we examined the efficacy of recombinant bacteria in various knockout mice. The efficacies of recombinant microbe treatment were not changed in CD8 T cell knockout mice (Fig. 5b). However, in mice that lacked NK cells, the efficacies of recombinant microbe treatment were particularly altered, reduced to a level below that of normal mice (Fig. 5c). NK cells are known as tumor suppressive cells. To confirm the role of NK cells in the tumor suppressive action of recombinant *Salmonella*, we isolated NK cells and treated them with recombinant *Salmonella*-harboring TNF-α. The NK cells were activated and secreted increased amounts of IFN-γ (Fig. 5d), and the activated NK cells induced cell lysis (Fig. 5e) and inhibited tumor growth in tumor-bearing mice (Fig. 5f). These results suggested that the efficacy of recombinant *Salmonella* is due to the dual effects of direct killing by the microbe harboring TNF-α and the induction of tumor-suppressive effects through activation of host NK cells.

Engineered *Salmonella* are induced anti-tumor effects in mice with antibiotics or vaccine or cisplatin

Elimination of bacterial infection by antibiotics and host immune responses would be disadvantageous to recombinant microbial treatment. To investigate the impact of antibiotics and immune responses on microbial tumor therapy, we examined the effect of antibiotics on bacterial treatments and tumor growth in mice that were vaccinated with normal *Salmonella* before inoculation with recombinant microbe. In vitro assay, antibiotics did not interfere with bacterial tumor therapy in cells (Fig 6a). In mice, treatment of microbe with antibiotics also reduced tumor growth (Fig. 6b). In mice that were vaccinated with normal *Salmonella*, host immune responses did not significantly affect tumor inhibition by recombinant *Salmonella* (Fig. 6c). These results suggested that antibiotics and host defense responses to subcutaneous inoculation do not interfere with the anti-tumor activity of recombinant *Salmonella*.

To test the efficacy of recombinant bacterial treatment in combination with existing therapies, we co-treated mice with chemotherapy (cisplatin) along with *Salmonella*. In mice, treatment with cisplatin and microbe was associated with a better effect than single treatment with cisplatin (Fig. 6d). These results suggested that microbial therapy could be used as an adjuvant to existing cancer therapies for more effective treatments of cancers.

Engineered *Salmonella* reduce growth of different types of tumors

To determine whether microbial therapy effected different tumor types, we transplanted TC-1, 4T-1, CT-26, and RENCA cells into mice. Although there was some variation in efficacy, microbial treatment reduced tumor growth and was associated with prolonged survival in all types of tumor models. These results suggested that, unlike existing treatments, microbial therapy can be applied to different types of tumors (Fig. 7)

Discussion

In the development of cancer treatments, safe, low-cost, and versatile agents are needed, not only as existing treatments but also as new therapeutics. Biological agents such as cell-based therapies, gene therapy, and immunotherapy have all been developed as potential cancer therapeutics. Recently, bacterial cell-based tumor treatments have been considered as a potential technology for tumor treatments and tumor vaccines.

Salmonella in particular has been shown to possess tumor-targeting properties and has been used as an agent and diagnostic tool (He et al. 2003). Tumor-targeted *Salmonella* exhibited tumor accumulation ratios in excess of 1,000:1 as compared to normal tissues (Bermudes et al. 2000), and many groups have used *Salmonella* strains in the development of anticancer agents (Toso et al. 2002). In the current study, we created a genetically engineered bacterial strain of *S. typhimurium* that harbored TNF-α as a

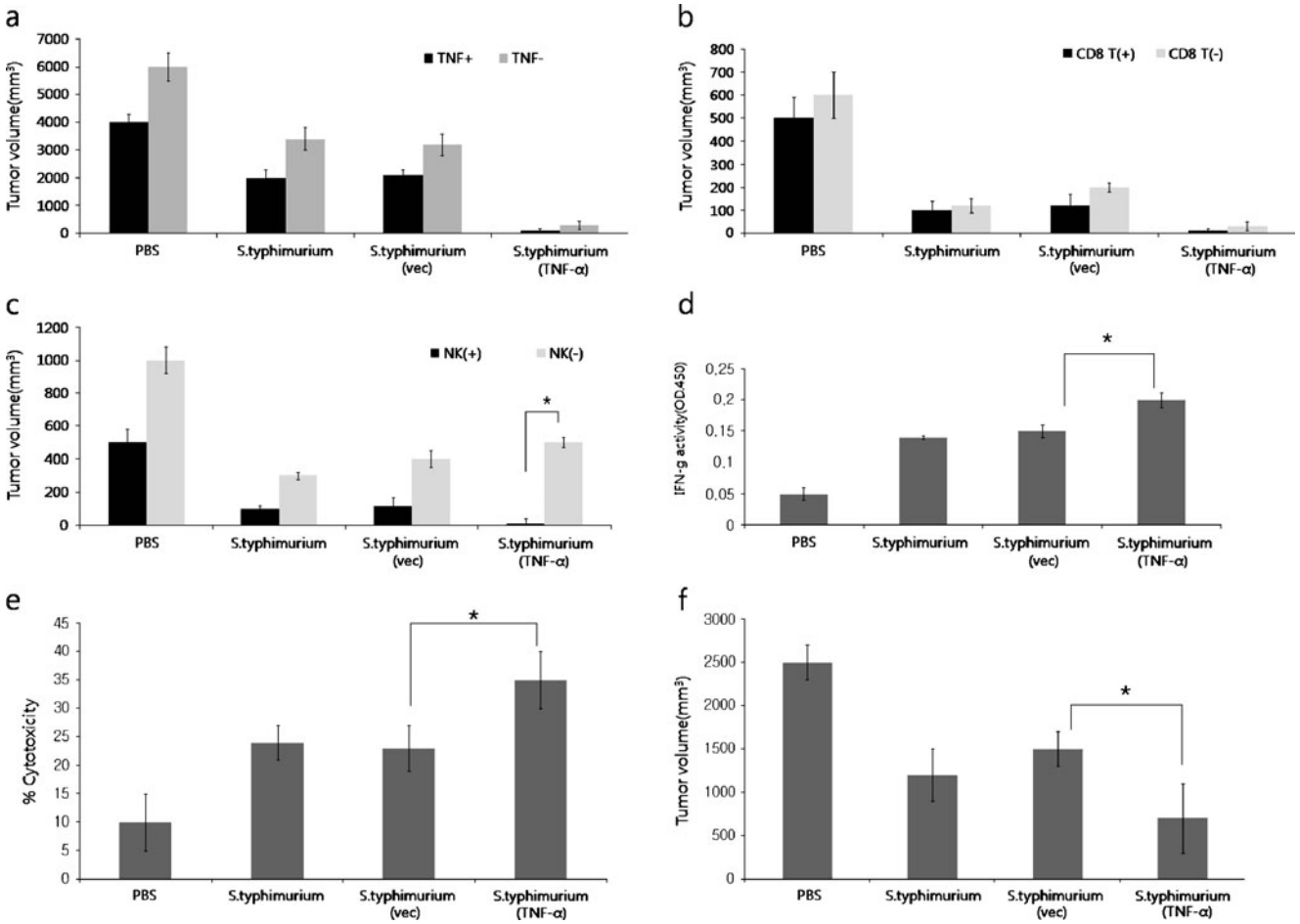

Fig. 5 NK cell activation by *S. typhimurium* harboring TNF-α induced tumor inhibition. **a** To test efficacy of TNF-α in treatment with *S. typhimurium* harboring TNF-α in mice, *Salmonella* strains were inoculated with 10 ng TNF-α protein in 100 μl PBS in C57BL6 mice, and the combinatory treatments with 1×10^8 *Salmonella* and TNF-α induced tumor suppression like *S. typhimurium* harboring TNF-α. **b** Growth prevention of B16F10 tumors in CD8 T cell-knockout mice by a subcutaneous inoculation. Tumor bearing mice were inoculated with 1×10^8 *S. typhimurium* harboring TNF-α on day 0 and 7. **c** Growth prevention of B16F10 tumors in NK-knock our mice by a subcutaneous inoculation of *S. typhimurium* harboring TNF-α. Tumor bearing mice were inoculated with *S. typhimurium* harboring TNF-α on day 0 and 7. $^*P<0.05$ as compared with the control groups. **d** Interferon gamma expressions were induced in NK cells by *S. typhimurium* harboring TNF-α in vitro. Isolated normal 1×10^6 murine NK cells were treated with PBS, and 1×10^8 bacterial groups. After incubation 24 h in RPMI medium with gentamycin, medium were analyzed with ELISA. **e** Direct melanoma cell killing activities of the NK cells activated by recombinant *Salmonella* in vitro. After NK cells cultured with PBS, and 1×10^8 bacterial groups for 24 h, isolated NK cells were co cultured with 1×10^5 B16F10 melanoma cells, after 24 h cultivation, tumor cell cytotoxicity were analyzed by CytoTox 96 non-radioactive cytotoxicity assay. $^*P<0.05$ as compared with the control groups. **f** Growth prevention of B16F10 tumors by a subcutaneous inoculation of NK cells activated with *S. typhimurium*-harboring TNF-α. Mice were inoculated with 1×10^5 B16F10 melanoma. Experimental animals in groups were each subcutaneously inoculated two times at 1 week intervals with 1×10^6 NK Cells with stimulated with *S. typhimurium* without secreting plasmid, *S. typhimurium* only (vec) and *S. typhimurium*-harboring TNF-α (TNF). Animals were examined daily until the tumor became palpable, after which its diameter was measured with microcalipers in two dimensions every other day. $^*P<0.05$ as compared with the control groups

potential anticancer agent. One of the requirements of this bacterial strain is that it has to be safe in normal cells, while localizing to various tumor cells, invading the tumor cells, and bombarding them with tumor suppressor proteins to induce tumor cell lysis. Examples of tumor suppressor proteins include VEGF, p53, p19, interferon, and other cytokines. Among these proteins, cytokines are of particular importance for their role in the regulation of the immune system. Limitations associated with systemic administration of cytokines include the short half-life of many cytokines and the severe side-effects are commonly observed with direct administration (Bocci 1988; Lissoni et al. 1996). TNF-α, for example, showed early promise as a tumor inhibitory molecule, with limitation in clinical trial because of its side effects, but local immunotherapy with this protein were promising (Terlikowski 2002). Our goal is to use bioengineering to reduce the side effects of TNF-α and increase its use an anti-cancer agent.

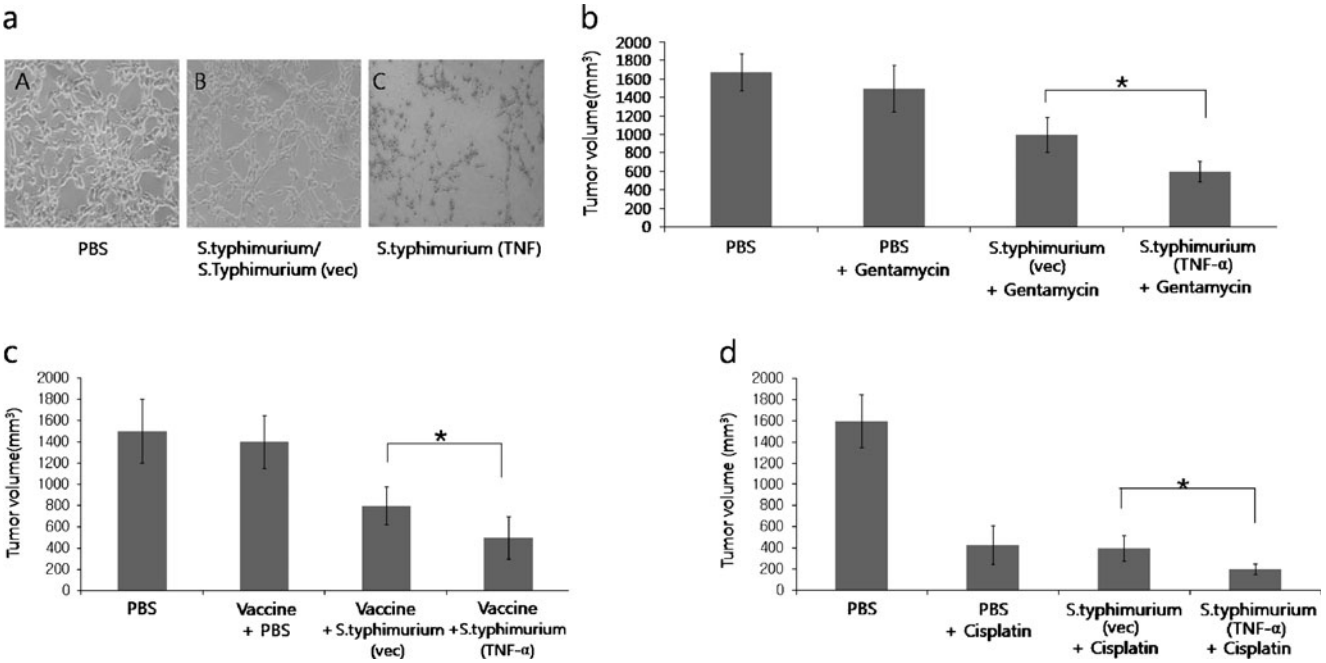

Fig. 6 Tumor inhibition of *S. typhimurium* harboring TNF-α with antibiotics or vaccine or cisplatin. **a** Suppression of tumor cell growth by the recombinant TNF-α proteins of *Salmonella* with antibiotics. Mouse melanoma B16F10 cells were treated with PBS (*A*), and *S. typhimurium* alone and *S. typhimurium* with a control vector (*B*), and *S. typhimurium* with pSSTM (*C*) in medium with *Salmonella*-specific antibiotics (gentamycin). **b** Growth prevention of B16F10 tumors by a subcutaneous inoculation of *S. typhimurium* harboring TNF-α with gentamycin. **c** Growth prevention of B16F10 tumors by a subcutaneous inoculation of *S. typhimurium* harboring TNF-α after pretreatment of *Salmonella*-vaccine. **d** To examine growth prevention of B16F10 tumors by a subcutaneous inoculation of *S. typhimurium* harboring TNF-α with existent therapeutic agents, bacterial administration with cisplatin was examined. Each cisplatins were inoculated with *S. typhimurium* harboring TNF-α on day 0 and 7. Experimental animals in groups were each subcutaneously inoculated two times at 1 week intervals with 10^8 attenuated *S. typhimurium* without secreting plasmid and *S. typhimurium* only. Animals were examined daily until the tumor became palpable, after which its diameter was measured with microcalipers in two dimensions every other day. $^*P<0.05$ as compared with the control groups

In the current study, *Salmonella* that was engineered to express TNF-α did not secrete cytokine in normal cells, but released the protein in tumor cells to reduce side effects of TNF-α. Our recombinant microbe specifically attached to and destroyed tumor cells without harmful cytokine variation and histological changes. These results suggest that *Salmonella*-harboring TNF-α would be a good alternative to TNF-α treatment.

Our results indicate that cytokine-expressing *S. typhimurium* could act as a good biological anticancer agent without the cytotoxicity of high-dose cytokine administration. The production of recombinant *Salmonella* would be

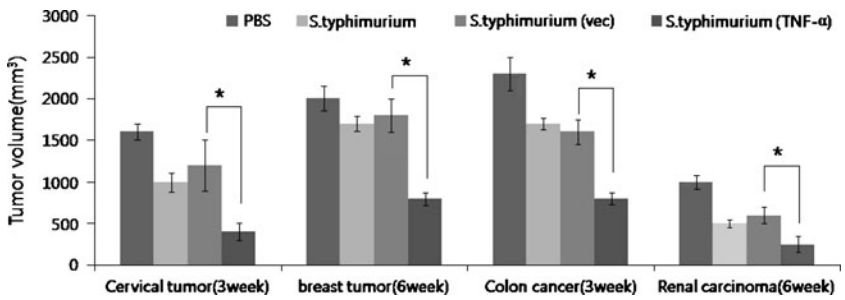

Fig. 7 Comparision of growth prevention of various tumors by a subcutaneous inoculation of *S. typhimurium* harboring TNF-α. Cervical tumor (TC-1), brest carcinoma (4T-1), colon cancer (CT-26), renal carcinoma (RENCA) cells were each transplanted in mice. Tumor-bearing mice were inoculated with *S. typhimurium* harboring TNF-α on day 0 and 7. Experimental animals in groups were each subcutaneously inoculated two times at 1 week intervals with 1×10^8 *S. typhimurium* alone, *S. typhimurium* with a control vector (vec) and *S. typhimurium* harboring TNF-α (TNF). Animals were examined daily until the tumor became palpable, after which its diameter was measured with microcalipers in two dimensions every other day. $^*P<0.05$ as compared with the control groups

convenient and easy, with a low cost and a short production time; and biological anticancer agents would be expected to have synergistic effects (bacterial cytotoxicity and immune induction of anticancer cytokines). We showed that engineered *Salmonella* induces NK cell activation and activated NK cells induce tumor inhibition.

Previous reports have shown that bacteria can function as gene delivery shuttles for transporting recombinant gene vectors (Pawelek et al. 1997; Dietrich et al. 2000; Huang et al. 2000). In the current study, recombinant *Salmonella* produced 100 pg of TNF-α per 10^9 cells. TNF-α was not secreted into the normal environment, but was released in tumor cells after bacterial invasion of the tumor cells.

We examined whole-cell lysates and culture supernatants by Western blot for the expression and secretion of the fusion protein. *Salmonella*-harboring TNF-α produced TNF-α in bacterial cell cultures, and did not secrete the protein into culture supernatants. Thus, bioengineering resulted in an agent that was safer and with potentially fewer side effects than the direct administration of TNF-α. TNF-α secretion was not detected in the blood of inoculated mice and histological side effects were not observed.

We tested the effects of recombinant *Salmonella* on tumor growth and survival of tumor-bearing mice following subcutaneous inoculation of 10^6 B16F10 melanoma cells. After 7 days, 10^8 bacterial cells were inoculated subcutaneously at the site of tumor transplantation. The inhibition of tumor growth was higher in mice that received recombinant TNF-α-expressing *Salmonella* as compared to bacterial strains that did not express cytokine.

When we examined whether cured mice possessed memory T-cells to counteract the tumor cells, we found that all mice developed tumors following second inoculation of tumor cells. Thus, the effects of bioengineered *Salmonella* were due to the temporary killing of tumor cells, and additional approaches are needed to induce tumor immune responses, such as the development of TNF-α fused to a tumor antigen.

Previous research showed that TNF-α activates the immature NK-free subset (Jewett and Bonavida 1993, 1994). The antitumor activity generated by selective activation of NK cells was studied in vitro and in vivo, and activation of NK cells induced inhibition of tumor growth (Hanna 1982). Other researchers reported that activation of NK cells retains the ability to inhibit hematogenous tumor metastasis (Hanna 1983). These results are applied in our recombinant microbe. We examined whether bioengineered *Salmonella* induced a tumor-suppressive environment through immune cell activation using knockout mice. We repeated the tumor cell transplantation experiments in NK cell-, MHC I-, MHC II-, CD8-, CD4-, IL-4-, IL-12-, and TNF-α-knockout mice (data not shown), and observed a significant effect in NK cell-knockout mice. In NK cell-knockout mice, the effects of the engineered bacteria against tumor growth were reduced as compared to the control groups. NK cells suppress tumor cells, and have been used as a cell-based therapy. Recombinant *Salmonella*-harboring TNF-α activated NK cells, and these activated NK cells induced tumor suppression. These results provided evidence that the effects of engineered bacteria are due to bacterial cytotoxicity and the induction of host immune responses with NK cell activation.

We were also interested in the efficacy of bioengineered *Salmonella* as an adjuvant to existing therapies, such as chemotherapy with cisplatin. Generally, anticancer agents like cisplatin and radiation do not interfere with bacterial activities (Salles and Calsou 1992; Salles et al. 1994; Bouayadi and Salles 1995). Thus, the possibility of using recombinant *Salmonella* as an adjuvant to tumor treatments was explored. If the microbe was synergistic with existing therapies, many factors would have to be considered, such as immune rejection, interference by the chemical agent, and/or biological interference.

We demonstrated that the efficacy of engineered *Salmonella* was not reduced by antibiotics and anti-bacterial immune responses following subcutaneous inoculation of the microbe. Our results suggested that the anti-tumor activity of recombinant *Salmonella* could be not restricted by host immunity and chemotreatment.

To examine the effect of combined treatment with engineered *Salmonella* and existing therapies, we demonstrated that the engineered microbe did not interfere with the efficacy of cisplatin and, in fact, significantly increased the therapeutic effect of these therapies on survival and tumor growth. Finally, we showed that bioengineered *Salmonella* was effective against various types of tumors. This might be related to the ability of the microbe to target tumors, not through a tumor-specific antigen, but by targeting the tumor environment. These results showed that our microbe could be used as a universal tumor treatment.

For more predictable clinical results, new drugs are prescribed along with existing drugs for patients (Braybrooke et al. 2005; Freytag et al. 2007; Saif et al. 2007; Baselga et al. 2009). Parallel development of new drugs is important, as is the development of adjuvants to increase the capabilities of existing anti-cancer drugs and improve outcomes for patients. Bacterial agents can be produced easily and cheaply, and engineered bacteria can be applied to a variety of tumors and in combination with different treatments.

In conclusion, we demonstrated that recombinant *Salmonella* harboring a cytokine expression vector (*S. typhimurium*-harboring TNF-α) mediates cytokine gene expression in vitro and exerts antitumor effects in mice. This microbe could be used as a versatile agent against many tumors and

as an adjuvant to existing therapies. Thus, *S. typhimurium*-harboring TNF-α could serve as an anticancer agent and provide a new, safe, and efficient way to treat cancer.

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Conflict of interest The authors declare that they have no conflict of interests.

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