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Chapter 7

Salmonella and Biotechnology

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Abstract

Salmonella strains have been actively studied as live carriers of heterologous antigens for a possible vaccine application. Especially, *Salmonella Typhimurium*, a facultative anaerobe, has been adapted as an antitumor agent capable of preferentially proliferating within tumors and inhibiting their growth. To enhance the cancer therapeutic efficacy of *S. Typhimurium*, combinations of gene-directed protein or microRNA therapies and auxotrophic strains of *S. Typhimurium* have been generated by genetic engineering. Until now, the idea of using bacteria including *Salmonella* in the treatments of cancer and other diseases has been considered a novel approach. Here, we describe this application based on *Salmonella* engineering for treatments of cancer or atopic dermatitis.

Keywords: *Salmonella*, cancer, atopic dermatitis, therapy, delivery system

1. Introduction

Salmonella strains have been used to prepare attenuated vaccines. These bacteria invade epithelial cells and secrete the internal protein of bacteria. Thus, *Salmonella* strains have been adapted as carriers for delivery of a recombinant antigen, therapeutic protein, or functional plasmid. After invading the intestinal epithelium, a modified *Salmonella* strain survives and replicates within antigen-presenting cells such as macrophages, mast cells, and dendritic cells.

Salmonella induces strong mucosal and cell-mediated immune responses against recombinant antigens [1, 2]. Recombinant proteins expressed by *S. Typhimurium* can be secreted and recognized by host immune cells with or without lysis of the bacteria. However, *Salmonella* replication within a membrane-bound vacuole inhibits processing of a recombinant protein by antigen-presenting cells [3]. Therefore, in a genetically modified *Salmonella* strain, a method for effective delivery of a recombinant protein carried by bacteria into the host is needed for the development of an effective therapeutic strain.

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Salmonella strains secrete recombinant proteins and introduce a heterologous protein into the extracellular environment. *Salmonella* strains use a type III secretion system (T3SS) to deliver cytoplasmic effector proteins into host cells [4]. In most T3SS-secreted proteins, *Salmonella* uses the N-terminal region for the signal for secretion of the target protein [5]. When several antigenic peptides are fused to the secretion domain of effector protein SopE of *S. Typhimurium* or YopE of *Yersinia enterocolitica*, the recombinant proteins are exported in a T3SS-dependent manner, resulting in activation of an immune response in mouse models [6, 7]. *S. Typhimurium* possesses two T3SSes encoded by *Salmonella* pathogenicity islands (SPIs) 1 and 2; SPI-1 is required for invasion of non-phagocytic epithelial cells, and SPI-2 for replication and survival in macrophages [4].

Salmonella has also been exploited as an antitumor agent that is capable of preferentially amplifying within a tumor and inhibiting its growth [8, 9]. In an effort to enhance therapeutic efficacy, this approach has been combined with a gene-directed enzyme/prodrug therapy [10]. For example, auxotrophic *S. Typhimurium* expressing prodrug-converting enzymes has been generated by transformation with a prokaryotic expression vector encoding herpes simplex virus thymidine kinase [11] or by chromosomal insertion of the *Escherichia coli* cytosine deaminase gene [12]. *Salmonella* has also been engineered for the transfer of prokaryotic and eukaryotic expression plasmids into host cells [13]. Oral administration of genetically modified *Salmonella* carrying a eukaryotic expression plasmid encoding interferon-gamma (IFN-γ) restores the production of this cytokine in the macrophages in mice [14]. When delivered orally to mice, *S. Typhimurium* carrying eukaryotic expression vectors encoding cytokines (i.e., interleukin-12 or GM-CSF) increases cytokine concentrations and exerts antitumor effects [15]. Thus, it should be feasible to use *Salmonella* strains transformed with eukaryotic expression vectors to deliver various effector molecules to cancer cells or skin inflammation sites, with the goal of enhancing therapeutic activity.

2. Medical application of *Salmonella* engineering

2.1. *Salmonella* strains are used as a carrier for delivery of a foreign protein or genetic material

Salmonella strains are considered good candidates as a vector for delivery of a foreign protein and/or plasmid(s). Attenuated *S. Typhimurium* strains are easy and cheap vector microbes to produce an antigen in comparison with any other synthetic protein delivery system and have been evaluated as vehicles for delivery of a plasmid expressing a heterologous antigen(s) to the host. Not only cytoplasmic expression of a recombinant protein in *S. Typhimurium* but also secretion or surface display of the target protein is a promising strategy for enhancing vaccine effects by improving recombinant antigen presentation in antigen-presenting cells. In one study, researchers used the T3SS signal from SipB, which possesses the domains for its secretion and outer membrane localization.

Many Gram-negative bacteria have a T3SS to deliver effector proteins into host cells, and the secretion signals of T3SS substrates have been used as carrier molecules for the delivery of foreign antigens or therapeutic molecules.

In an animal experiment, oral administration of attenuated *S. Typhimurium* bearing therapeutic plasmids showed that this strain secretes and surface-displays the SipB tetanus toxin and induces a strong antigen-specific immune response in mice.

Oral vaccination of mice with attenuated *S. Typhimurium* carrying T3SS-based delivery plasmids may increase the recombinant protein presentation in antigen-presenting cells, resulting in induction of recombinant protein-specific immune responses in mice. These findings suggest that the N-terminal domain of SipB can serve as a signal sequence for the surface display and secretion of heterologous proteins.

However, successful T3SS-mediated antigen delivery is restricted by several factors, including the size, folding, stability, and structure of a recombinant protein.

When a highly virulent *Salmonella* strain (*S. Typhimurium* UK-1) is transformed with a cytokine (IL-12)-expressing plasmid, this live, wild-type pathogen has been shown to work as a vaccine strain without any other biological or genetic attenuating processes.

Wild-type pathogenic *S. Typhimurium* UK-1 carrying an IL-12-expressing plasmid induces protection against a lethal dose of normal wild-type *Salmonella* [16]. These results also revealed that a wild-type *Salmonella* strain bearing a plasmid for secretion of IL-12 may be considered an alternative approach to the development of intracellular bacterial vaccines, without the inconvenience of time-consuming attenuation procedures.

2.2. Engineered *Salmonella* has therapeutic effects on cancer

In the field of anticancer therapeutic agents, biological modalities such as cell therapy, gene therapy, and antibody-related immunotherapy have been developed as possible candidates for cancer therapeutics. In addition to these new candidates, bacterial therapy is believed to be a promising technology of tumor treatments and tumor vaccines. This kind of bacterial therapy is safer, less expensive, and more versatile than other biological cancer treatments. These therapeutic bacteria could be produced cheaply. Moreover, *Salmonella* is thought to be a good anticancer therapeutic agent and has shown tumor-targeting properties and tumor-suppressing effects in some studies [17]. Tumor-targeted *Salmonella* has a tumor accumulation ratio greater than 1000:1 as compared to healthy tissues [9], and many research groups have used *Salmonella* strains for the development of anticancer agents [18]. In one study, a genetically engineered strain of *S. Typhimurium* expressing TNF-α was tested as a melanoma-suppressing agent. This *Salmonella* was attenuated for safety toward healthy cells and was specifically localized to and invaded various melanoma cells while bombarding them with tumor necrosis factor (TNF) proteins to induce tumor cell apoptosis. VEGF, p53, p19, IFNs, and other cytokines have been tested as tumor-suppressor proteins in *Salmonella* vector systems. Although systemically administered cytokines have short half-life and severe side effects after direct administration, cytokines are often used for regulation of the immune system and for tumor cell lysis [19]. For *Salmonella* cancer therapy, researchers engineered *Salmonella* expressing the TNF-α protein—a potent antitumor molecule that normally has limitations because of its side effects—to reduce the adverse effects via tumor-specific local immunotherapy [20].

In our test of *Salmonella*-based TNF-α therapy, the targeted recombinant TNF-α from bacteria did not induce histological changes in various tissues and cytokine upregulation such as severe inflammations after local administration of recombinant *Salmonella*. Some researchers reported that cytokine-expressing *S. Typhimurium* could act as a good biological anticancer agent without the cytotoxicity of high-dose cytokine administration. The production of genetically modified *Salmonella* would be convenient and easy, with a low cost and a short manufacture period; such biological anticancer agents are expected to have synergistic effects (bacterial cytotoxicity and immunostimulation by anticancer cytokines). In addition, bacteria can function as gene delivery shuttles for transporting recombinant gene vectors [11]. According to the latest studies, recombinant *Salmonella* produces 100 pg of TNF-α per 10^9 cells. TNF-α is not secreted into the normal environment, but is released into tumor cells after bacterial invasion of these cells. Accordingly, genetically modified *Salmonella* carrying a cytokine expression vector (e.g., *S. Typhimurium* expressing TNF-α) is known to express cytokines in melanoma cells and to suppress tumor growth in mice with melanoma, colon cancer, or breast cancer. Therefore, *S. Typhimurium* expressing TNF-α may serve as a therapeutic agent against various tumors and as an adjuvant to existing cancer therapies such as chemotherapy, radiotherapy, and immunotherapy. These findings suggest that *Salmonella* carrying a cytokine expression vector can act as a new, safe, and efficient anticancer agent. In particular, to determine the cytotoxic effects of genetically modified *Salmonella*, B16F10 melanoma cells were treated with *S. Typhimurium* expressing TNF-α. The *S. Typhimurium* expressing TNF-α invaded tumor cells at a higher invasion rate (50%) than did a naïve *Salmonella* strain and lysed the melanoma cells [21]. These results indicate that genetically modified *Salmonella* expressing TNF-α specifically lyses B16F10 melanoma cells in contrast to naïve *Salmonella* strains (**Figure 1a**) and that the engineered *S. Typhimurium* expressing TNF-α induces caspase activation for tumor cell lysis and inhibited tumor growth in tumor-bearing mice (**Figure 1b**).

Additionally, a *Salmonella*-based cancer therapy may not be compatible with antibiotics like gentamicin, and host immune responses and environments conducive to bacterial killing are

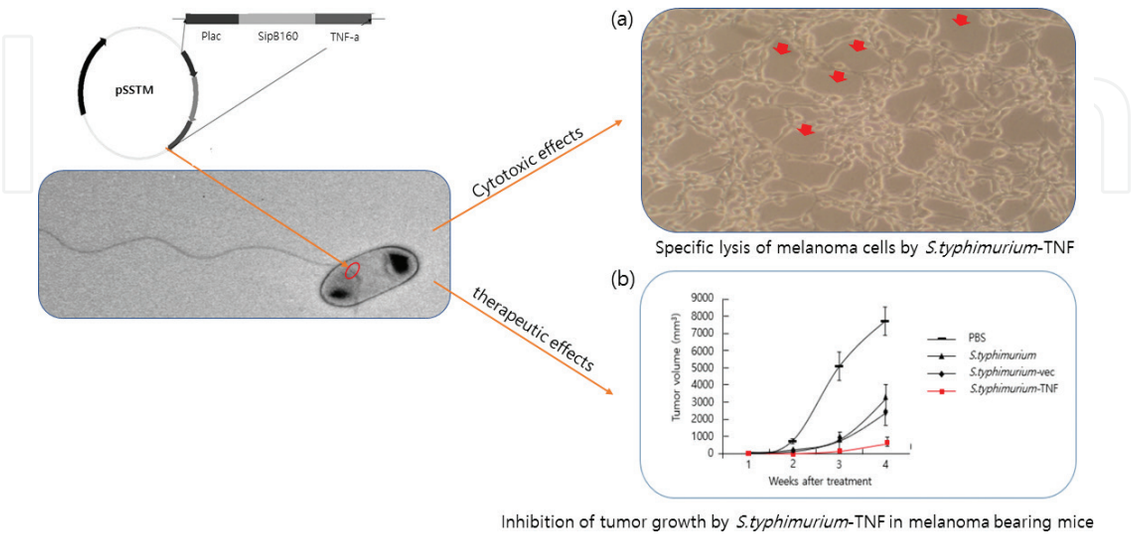


Figure 1. Construction and effects of the recombinant *Salmonella Typhimurium* containing TNF-α.

likely to be disadvantageous for *Salmonella*-based cancer treatment. In some studies, researchers investigated the impact of antibiotics on a *Salmonella*-based cancer therapy. Tumor-bearing mice were treated with *Salmonella* expressing TNF-α and an antibiotic (gentamicin). Another group of mice was vaccinated with attenuated *Salmonella*, received a transplant of melanoma cells, and was then examined for the tumor inhibitory effect of *Salmonella* expressing TNF-α. In an in vivo assay, gentamicin did not interfere with *Salmonella*-mediated therapy of tumor cells (**Figure 2a**). In tumor-bearing mice, treatment with *Salmonella* and cisplatin also inhibited tumor growth (**Figure 2b**). In mice that were vaccinated with dendritic cells, host immune responses did not suppress tumor inhibition by *Salmonella* expressing TNF-α (**Figure 2c**). In treatment with *Salmonella* and radiation, *Salmonella* expressing TNF-α also inhibited tumor growth (**Figure 2d**). These results showed that the antitumor activity of subcutaneous treatment with *Salmonella* expressing TNF-α is not suppressed by antibiotics and host immune responses in mice.

Other studies were conducted on a vaccine based on recombinant *Salmonella* expressing human papilloma virus antigens [22]. This recombinant *Salmonella* was constructed from *Salmonella enterica* serovar *Typhimurium* expressing a fusion protein consisting of the SipB protein from *Salmonella* and the HPV16 E7 protein derived from human papillomavirus for tumor-suppressing effects. The genetically modified *Salmonella* expressing SipB-E7 was tested in a cervical cancer model. In cervical cancer TC-1-bearing mice, *Salmonella* expressing SipB-E7 induced cytotoxicity and slowed tumor growth after oral inoculation. Moreover, in the mouse model of cervical cancer, orally administered *Salmonella* expressing SipB-E7 induced cytokines IFN-γ and TNF-α and prolonged survival compared with the control group (naïve *Salmonella* or PBS-treated groups). These results revealed that *Salmonella* expressing fusion protein SipB160-E7 may be a candidate cancer therapeutic agent. Yoon et al. took advantage of a genetically engineered *Salmonella* strain as a candidate tumoricidal modality; to enhance tumor-suppressing effects, *S. Typhimurium* was designed to carry eukaryotic expression

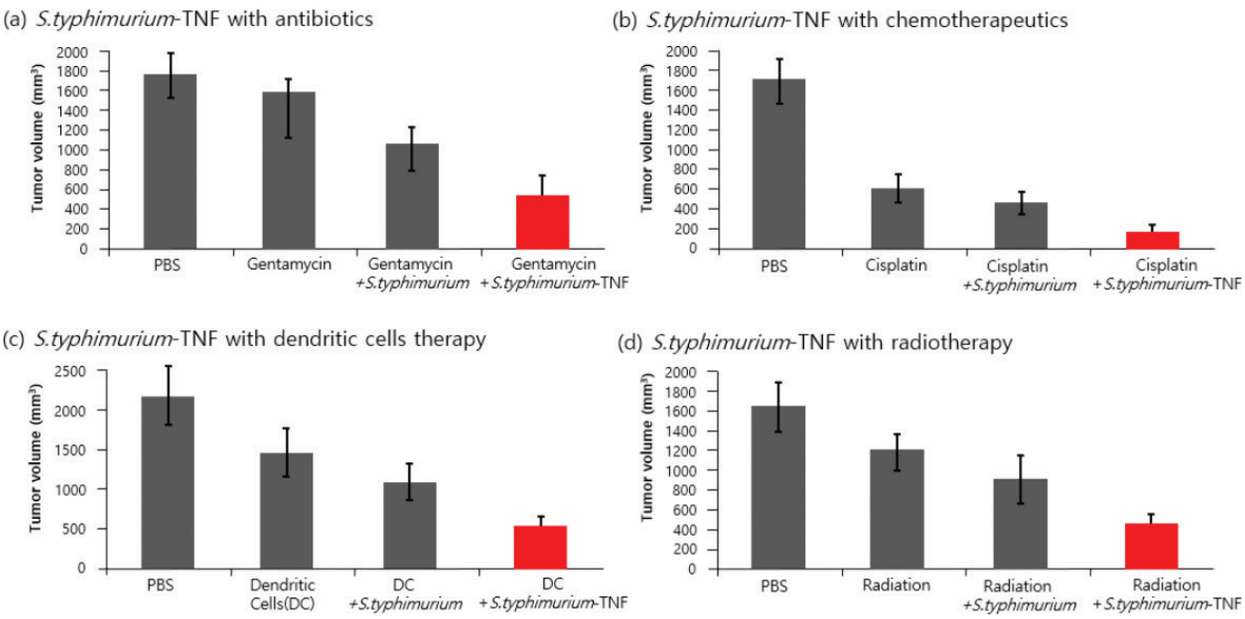


Figure 2. Tumour inhibition of *S. Typhimurium* containing TNF-α with antibiotics or vaccine or cisplatin.

plasmid expressing the Flt3 ligand (Flt3L) [23]. *Salmonella* carrying the Flt3L expression vector exerted antitumor effects against B16F10 melanoma cells in vitro. When the engineered *Salmonella* was injected locally into the tumor region, *S. Typhimurium* with the Flt3L expression vector inhibited tumor growth more effectively relative to control groups. Nonetheless, in the mice cured of melanoma after treatment with recombinant *Salmonella*, there was no induction of tumor immunity mediated by memory antitumor lymphocytes because there was no protective responses against a tumor rechallenge.

Compared to radiation alone, a combination therapy, *Salmonella* with γ-radiation, confers radiosensitization onto cancer cells by inducing apoptotic cell death [24]. *Salmonella* infection induces apoptosis via caspase 3 and Bcl2 in tumor cells. In addition, tumor growth is suppressed by this combined therapy pointing to possible new versions of radiation therapy against melanoma. Overall, cancer radiation therapy is significantly improved by the use of bacteria. For this reason, our findings indicate that bacteria may help to increase effectiveness of cancer radiation therapy in the future.

2.3. Engineered *Salmonella* induced therapeutic effects on atopic dermatitis

Salmonella-based therapy has been examined as a therapeutic agent for allergic diseases. Excessive Th2-biased immune responses are related to the pathogenesis of allergic diseases. Macrophage-derived chemokine (MDC) is directly related to Th2-associated atopic dermatitis, and MDC concentration is significantly elevated in the serum of patients. MDC has been studied as a marker of severity of atopic dermatitis. Yoon et al. tested genetically modified *Salmonella* as a gene therapy tool to treat atopic dermatitis with bacteria expressing specific microRNA [25]. To suppress the MDC gene for atopic dermatitis therapy, a *S. Typhimurium* strain was constructed that carries a plasmid expressing MDC microRNA. The engineered *Salmonella* strain bearing the microRNA-expressing plasmid (ST-miR-MDC) was used for an in vitro knockdown of MDC in human mast cells [26]. ST-miR-MDC was shown to significantly downregulate the MDC gene in activated human mast cells in vitro. In an atopic-like animal model, strain ST-miR-MDC downregulated IL-4 and IgE expression and upregulated IFN-γ. Strain ST-miR-MDC also suppressed Th17 in the atopic-like animal model (**Figure 3**).

In addition, orally administered strain ST-miR-MDC induced skin regeneration and hair regrowth in atopic-like mice, but control mice did not show these effects (**Figure 4a**). Pruritus

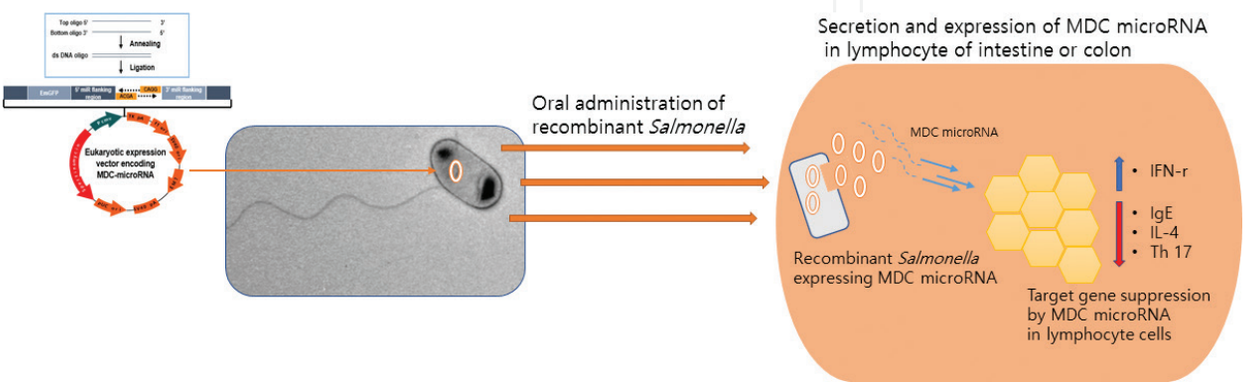


Figure 3. Construction of the recombinant *Salmonella Typhimurium* expressing miRNA-MDC.

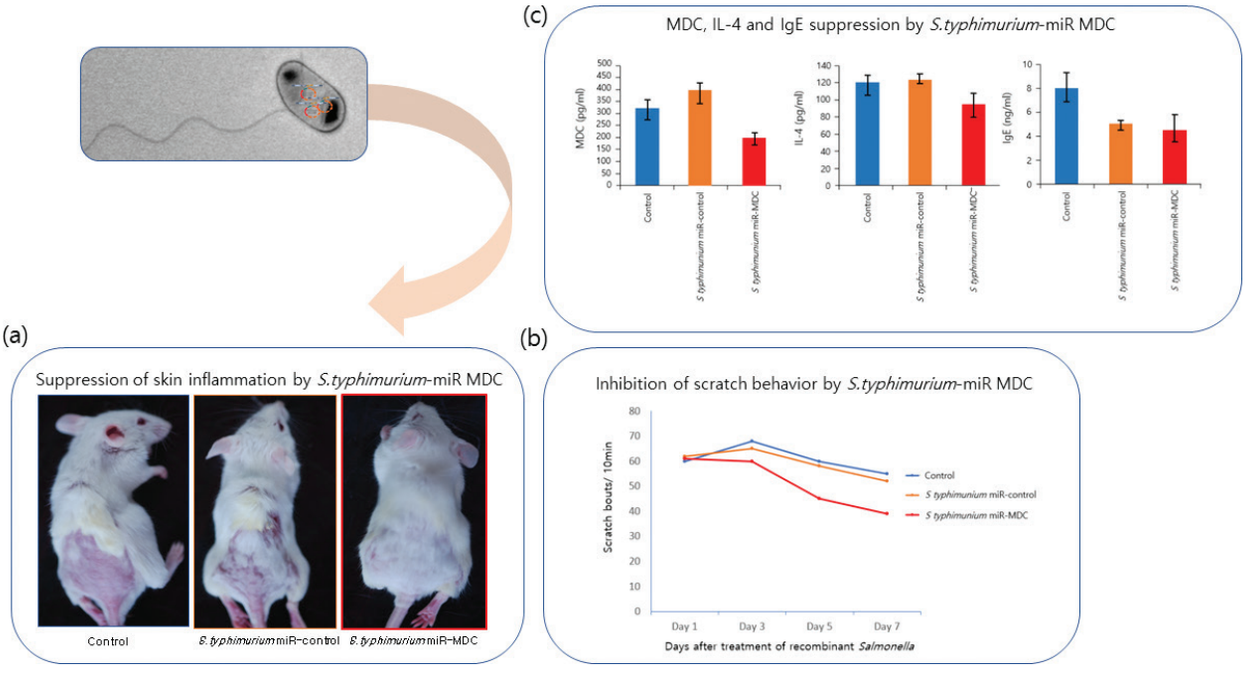


Figure 4. Improvement of the symptoms of atopic dermatitis by oral administration of *S. Typhimurium* miR-MDC in mice.

is one of the major symptoms of atopic dermatitis, and the ST-miR-MDC strain inhibited the scratching behavior of mice. The total scratching counts in the ST-miRCCL22-treated group were significantly lower than those among the mice treated with PBS or ST-miR-control (**Figure 4b**). This strain induced histological changes in the skin tissues of atopic-like mice after oral administration of the engineered *Salmonella*. Strain ST-miR-MDC reduced skin inflammation reactions and reduced cytokine IL-4, MDC, and IgE on mouse blood (**Figure 4c**).

These results indicate that *Salmonella* combined with a targeted microRNA delivery system may be a good candidate for the development of a therapeutic agent against atopic dermatitis.

3. Conclusions

To date, the idea of using bacteria, including *Salmonella* therapy, has been considered a novel approach. *S. Typhimurium* bearing a cytokine-expressing plasmid exerts an antitumor effect on melanoma or anti-inflammatory effects in an atopic-like mouse model.

The engineered *Salmonella* has been designed to target cancer cells, promote a tumor-suppressive environment, and increase the efficacy of existing cancer treatments, including chemotherapy, radiotherapy, and cell therapy.

Especially, *Salmonella* expressing microRNA has been used in vivo to knockdown a target gene and shows modulation of immune responses in a mouse disease model.

These results suggest that genetic engineering of *S. Typhimurium* may be an efficient method of delivery of cytokines or microRNA for therapeutic purposes.

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