Targeted Therapy with a *Salmonella Typhimurium* Leucine-Arginine Auxotroph Cures Orthotopic Human Breast Tumors in Nude Mice

Ming Zhao,[1] Meng Yang,[1] Huaiyu Ma,[1] Xiaoming Li,[1] Xiuying Tan,[1] Shukuan Li,[1] Zhijian Yang,[1] and Robert M. Hoffman[1,2]

[1]AntiCancer, Inc. and [2]Department of Surgery, University of California, San Diego, California

Abstract

We report here a modified auxotrophic strain of *Salmonella typhimurium* that can target and cure breast tumors in orthotopic mouse models. We have previously reported development of a genetically modified strain of *S. typhimurium*, selected for prostate tumor targeting and therapy *in vivo*. The strain, termed *S. typhimurium* A1, selectively grew in prostate tumors in xenograft models causing tumor regression. In contrast, normal tissue was cleared of these bacteria even in immunodeficient athymic mice with no apparent side effects. A1 is auxotrophic (leucine-arginine dependent) but apparently receives sufficient nutritional support only from tumor tissue. The ability to grow in viable tumor tissue may account, in part, for the unique antitumor efficacy of the strain. In the present report, to increase tumor-targeting capability of A1, the strain was reisolated after infection of a human colon tumor growing in nude mice. The tumor-isolated strain, termed A1-R, had increased targeting for tumor cells *in vivo* as well as *in vitro* compared with A1. Treatment with A1-R resulted in highly effective tumor targeting, including viable tumor tissue and significant tumor shrinkage in mice with s.c. or orthotopic human breast cancer xerographs. Survival of the treated animals was significantly prolonged. Forty percent of treated mice were cured completely and survived as long as non–tumor-bearing mice. These results suggest that amino acid auxotrophic virulent bacteria, which selectively infect and attack viable tumor tissue, are a promising approach to cancer therapy. (Cancer Res 2006; 66(15): 7647-52)

Introduction

It has been known for ~60 years that anaerobic bacteria can selectively grow in tumors (1–16). The conditions that permit anaerobic bacterial growth (i.e., impaired circulation and extensive necrosis) are found in many tumors. Several approaches to developing tumor-therapeutic anaerobic bacteria have been described. Yazawa et al. (14, 15) showed that the anaerobic bacterium *Bifidobacterium longum* could selectively grow in the hypoxic regions of solid tumors. Dang et al. (17) created a strain of *Clostridium novyi* depleted of its lethal toxin and showed that i.v. administered *C. novyi* spores germinated within the avascular regions of tumors in mice and destroyed surrounding viable tumor cells (17). The main efficacy of these anaerobic bacteria was in combination with chemotherapy (17). In immune competent mice or rabbits, the extensive destruction of tumors, coupled with an induced immune response, leads to complete eradication of tumors and cure in ~30% of the animals (18). In the other animals, the tumor regrows from a well-vascularized rim that is resistant to *C. novyi-NT* infection. Cures can also be achieved more often in mice by combining *C. novyi-NT* with radiation (19). In initial experiments with *C. novyi-NT*, 10% to 25% of animals with large tumors died after receiving therapeutic doses of *C. novyi-NT* spores (17). However, toxicity could be effectively controlled by simple hydration (20).

The facultative anaerobe *Salmonella typhimurium* was first attenuated by purine and other auxotrophic mutations to be used for cancer therapy (12, 21, 22). These bacteria replicated in the tumor to >1,000-fold compared with normal tissues (12). *Salmonella* lipid A was also genetically modified by disrupting the *msbB* gene to reduce septic shock (12). Melanomas in mice treated with the *Salmonella msbB* mutant were 6% the size of tumors in untreated controls (12). However, these *Salmonella* variants did not cause tumor regression or eradication, only growth inhibition. The *S. typhimurium* with attenuated lipid A has been evaluated in a phase I clinical trial (23). However, within the tumors, most bacteria were located in necrotic tissue as large colonies far from functional vasculature. It was therefore thought that *S. typhimurium* only survives in tissue that becomes necrotic (24). It was observed that *S. typhimurium* are attracted to tumor cell cylindroids *in vitro* and accumulate in the central region of large cylindroids. Both bacterial growth and chemotaxis were significantly greater in large cylindroids, suggesting that quiescent tumor cells secrete bacterial chemoattractants and the presence of necrotic and quiescent tumor cells enable *S. typhimurium* to replicate in tumor tissue (25).

Various species of bacteria labeled by green fluorescent protein (GFP) or luciferase and injected i.v. in live animals were observed to selectively replicate in solid tumors and in their metastases, including tumors of the breast, prostate, brain, and fibrosarcoma (26). Antitumor efficacy was not observed in these experiments. However, this approach is promising for labeling the tumors for imaging.

We have developed previously (27) a genetically modified bacterial strain of *S. typhimurium*, auxotrophic for leucine and arginine, which also expresses GFP, termed *S. typhimurium* A1. When introduced i.v. or intratumorally, A1 invaded and replicated intracellularly in PC-3 prostate cancer cells *in vivo* as well as *in vitro*. When A1 was injected intratumorally, the tumor completely regressed by day 20. There were no obvious adverse effects on the host when the bacteria were injected i.v. or intratumorally. The *S. typhimurium* A1 strain grew throughout the tumor, including viable malignant tissue. This result is in marked contrast to the anaerobic bacteria evaluated previously for cancer therapy that were confined to necrotic areas of the tumor as discussed above. The ability to grow in viable tumor tissue may account, in part, for the unique antitumor efficacy of the A1 strain.

Requests for reprints: Robert M. Hoffman, AntiCancer, Inc., 7917 Ostrow Street, San Diego, CA 92111. Phone: 858-654-2555; Fax: 858-268-4175; E-mail: all@anticancer.com.

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In the present study, the A1 strain was reisolated from A1-targeted tumor tissue *in vivo*. The idea was to increase the tumor-targeting capability of the bacteria. As a consequence of this selective step, the tumor cell targeting of the reisolated A1 increased *in vivo* as well as *in vitro*. The reisolated A1 bacteria, termed A1-R, administered i.v., caused human breast cancer regression and cures in nude-mouse models.

Materials and Methods

***GFP* gene transfection of *S. typhimurium* A1.** *S. typhimurium* (American Type Culture Collection 14028, Rockville, MD) was grown at 37°C to midlogarithmic phase in Luria-Bertani (LB) medium and harvested at 4°C. Cells (2.0×10^8) in 40 μL glycerol (10%) were mixed with 2 μL pGFP vector (Clontech, Palo Alto, CA) and placed on ice for 5 minutes before electroporation with a Gene Pulser apparatus (Bio-Rad, Hercules, CA) according to the manufacturer's instructions. Electroporation was done at 1.8 kV with the pulse controller at 1,000-Ω parallel resistance (27).

Reisolation of *S. typhimurium* A1. *S. typhimurium* A1 auxotrophs expressing GFP were reisolated as follows: the A1 bacteria were injected into the tail vein of a HT-29 human colon tumor-bearing nude mouse. Three days after infection, the tumor tissue was removed from the infected mouse. The tumor tissue was then homogenized and diluted with PBS. The resulting supernatant of the tumor tissue was cultured in LB agar plates at 37°C overnight. The bacteria colony with the brightest green fluorescence was picked up and cultured in 5 mL LB medium. This strain was termed A1-R.

Adherence and invasion assay. Red fluorescent protein (RFP)–labeled HT-29 human colon cancer cells were grown in 24-well tissue culture plates to a density of $\sim 10^4$ cells per well. A1-R bacteria were grown to late-log phase in LB broth as described previously (27). The bacteria were diluted in cell culture medium (1×10^6), added to the tumor cells, and placed in an incubator at 37°C. After 60 minutes, the cells were rinsed five times with 1 to 2 mL PBS. Adherent bacteria were released by incubation with 0.2 mL 0.1% Triton X-100 for 10 minutes. LB broth (0.8 mL) was then added, and each sample was vigorously mixed. Adherent bacteria were quantified by plating in order to count colony-forming units (CFU) on LB agar medium. To measure invasion of bacteria, the bacterially infected cancer cells were rinsed five times with 1 to 2 mL PBS and cultured in medium containing gentamicin sulfate (20 μg/mL) to kill external but not internal bacteria. After incubation with gentamicin for 12 hours, the cells were washed once wih PBS, and the viable intracellular bacteria were evaluated by fluorescence microscopy.

Surgical orthotopic implantation of breast tumors. Tumor fragments ($1\ mm^3$) from the MARY-X human breast tumor xenograft (28), grown s.c. in nude mice, were implanted by surgical orthotopic implantation in the mammary fat pad in nude mice. 8-0 surgical sutures are used to penetrate the tumor pieces to the fat pad. The incision in the skin was closed with a 7-0 surgical suture in one layer. The animals were kept under isoflurane anesthesia during surgery (29, 30).

Analysis of infection *in vivo*. At various time points after injection of bacteria, tissue samples were obtained from lung, liver, spleen, kidney, heart, and tumor. Normal tissues and tumors were excised and weighed and observed under fluorescence microscopy to determine the extent of bacterial infection. For biodistribution studies, CFU of bacteria in the tumors and normal mouse tissues were determined at different time points after inoculation by harvesting these tissues and homogenizing and plating supernatants on nutrient media. Tissues were also prepared for standard frozen sectioning and H&E staining for histopathologic analysis (27).

Bacterial treatment of tumor-bearing mice. The A1-R bacteria were grown overnight on LB medium and then diluted 1:10 in LB medium. Bacteria were harvested at late-log phase, washed with PBS, and then diluted in PBS. Bacteria were then injected into the tail vein of nude mice (5×10^7 CFU/100 μL PBS).

Results and Discussion

Enhanced tumor-cell adherence and invasion *in vitro* by *S. typhimurium* A1 reisolated from tumors (A1-R). *S. typhimurium* A1, expressing GFP, was passaged by injection in nude mice transplanted with the human colon tumor HT-29. Bacteria, expressing GFP, isolated from the infected tumor were then cultured. The ability of reisolated A1, termed A1-R, to adhere to tumor cells was evaluated compared with the parental A1 strain *in vitro*. The number of A1-R bacteria which attached to HT-29 tumor cells was ~ 6 times higher than parental A1 bacteria (Fig. 1*A*). The bacteria that could not be washed from the tumor cells invaded and replicated in the tumor cells. Fluorescence imaging showed that A1-R, which expressed GFP, entered and grew in RFP-expressing tumor cells, to a greater extent than the parental A1 (Fig. 1*B* and *C*). Thus, passage of A1 through the HT-29 tumor increased the ability of A1 to adhere to and invade tumor cells.

Targeting A1-R to human breast cancer in nude mice. A1-R bacteria targeted, colonized, and replicated in the human MARY-X breast tumors growing s.c. in nude mice as observed by fluorescence imaging on day 2 after i.v. injection (Fig. 2*A* and *B*). Subsequently, the bacteria grew throughout the tumor, including the surface of the tumor as well as deeper and central areas, by day 5 after i.v. infection. Bacterial growth within the tumor was observed by fluorescence imaging of the infected tumor tissue in transverse sections (Fig. 2*C* and *D*). The wide distribution of bacteria within the tumor, including viable tissue, is a major advance in bacterial tumor therapy. In nude mice with orthotopic MARY-X human breast tumors, A1-R bacteria also targeted and accumulated within the tumor after i.v. administration.

Destruction of tumor tissue by reisolated *S. typhimurium* A1. A1-R destroyed viable tumor tissue (Fig. 2*E-G*). The bacteria effectively targeted tumor tissue within the vascular regions, subsequently destroying viable tumor tissue (Fig. 2*E-G*). The rich vascularity of the MARY-X tumor may act as a delivery vehicle for the bacteria within the tumor after i.v. injection.

Efficacy of A1-R on tumor growth. Treatment with A1-R resulted in significant tumor shrinkage in nude mice with s.c. MARY-X human xerographs. Bacteria (5×10^7 CFU/100 μL) were inoculated i.v. in MARY-X-bearing nude mice. Tumor growth was monitored by caliper measurement in two dimensions. The infected tumors regressed by day 5 after infection, and complete regression occurred by day 25 (Fig. 3*A* and *B*). In orthotopic models of MARY-X, A1-R treatment also led to tumor regression following a single i.v. injection of A1-R (Fig. 4*A* and *B*). The destruction of the tumor in treated mice was visualized by whole-body imaging (Fig. 4*A*). The difference in tumor volume between the treated group, which showed quantitative regression, and the control was statistically significant ($P < 0.05$; Fig. 4*B*).

Survival efficacy of A1-R. The survival of the A1-R-treated animals was prolonged with a 50% survival time of ~ 13 weeks compared with 5 weeks of control animals (Fig. 3*C*). Forty percent of the mice survived as long as control non–tumor-bearing mice (Fig. 3*C*). In the cured animals, tumors were completely eradicated with no regrowth. In contrast, the parental *S. typhimurium* A1 was less effective than A1-R. Tumor growth was only slowed after A1 i.v. injection and not eradicated (data not shown).

Safety of A1-R. There were no obvious toxic effects on the host even with 5×10^7 A1-R injected i.v. The mice tolerated the infection and could survive as long as nontreated mice. A1-R only replicates and persists within the microenvironment of the tumor and cannot continuously survive outside the tumor site. Normal tissues, such as liver and spleen, were initially infected by the bacteria after injection. However, the bacteria colonies failed to

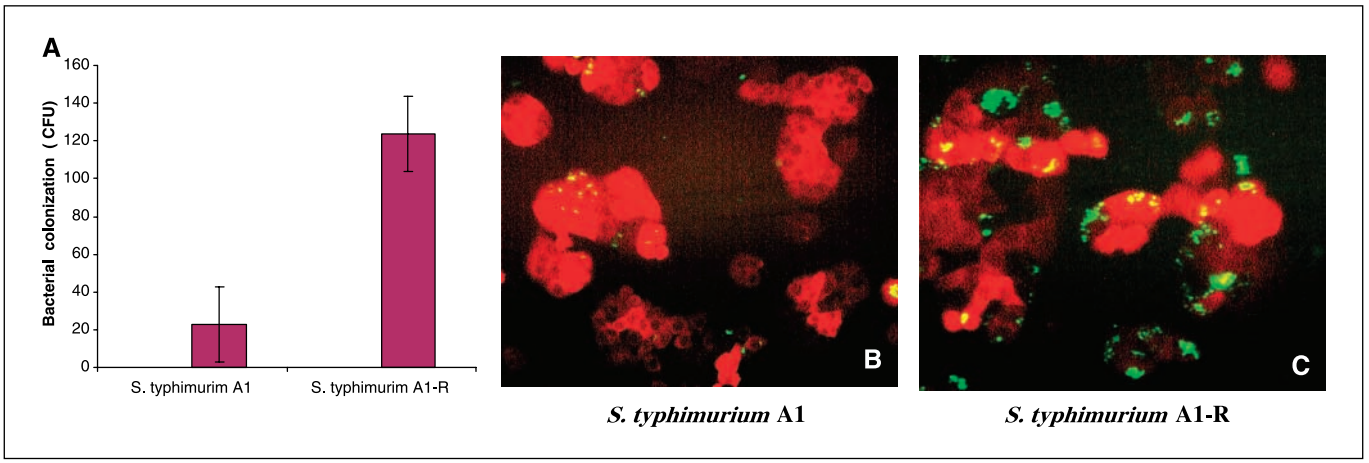

Figure 1. Tumor targeting of *S. typhimurium* A1 and A1-R *in vitro*. *A,* RFP-labeled HT-29 human colon cancer cells were grown in 24-well tissue culture plates. *S. typhimurium*-GFP, grown to late-log phase, were diluted in cell culture medium (1×10^6), added to the tumor cells, and placed in an incubator at 37°C. After 60 minutes, the cells were rinsed. Adherent bacteria were released and then quantitated by plating for CFU on LB agar medium. The number of A1-R bacteria attached to tumor cells was ~6 times higher than the parental A1 bacteria. *B* and *C,* to measure invasion of bacteria, the cancer cells were first rinsed five times with PBS, and then the cells were cultured in medium containing gentamicin sulfate (20 μg/mL) to kill external but not internal bacteria. After incubation with gentamicin for 12 hours, the cancer cells were washed once with PBS, and the viable GFP-expressing intracellular bacteria were observed by fluorescence microscopy. A1-R (*C*) bacteria invaded and grew in tumor cells to a greater extent than the parental A1 (*B*). Bar, 58 μm.

progressively grow in the normal tissue and subsequently completely disappeared from normal tissue by day 15 after infection (data not shown).

This new approach to targeting of viable tissue tumors by facultative anaerobic bacteria, in contrast to previous strategy of targeting hypotic and nonviable areas of the tumors by anaerobic bacteria, has significant promise. An important distinction of the present studies, unlike other approaches of bacterial therapy of cancer using obligate anaerobes, is that A1-R can cure tumors when used alone. The studies using obligate anaerobic bacteria required combination with chemotherapy to effect cures. This requirement for combination chemotherapy may be due to the inability of the obligate anaerobic bacteria to grow in oxygenated viable tumor tissue.

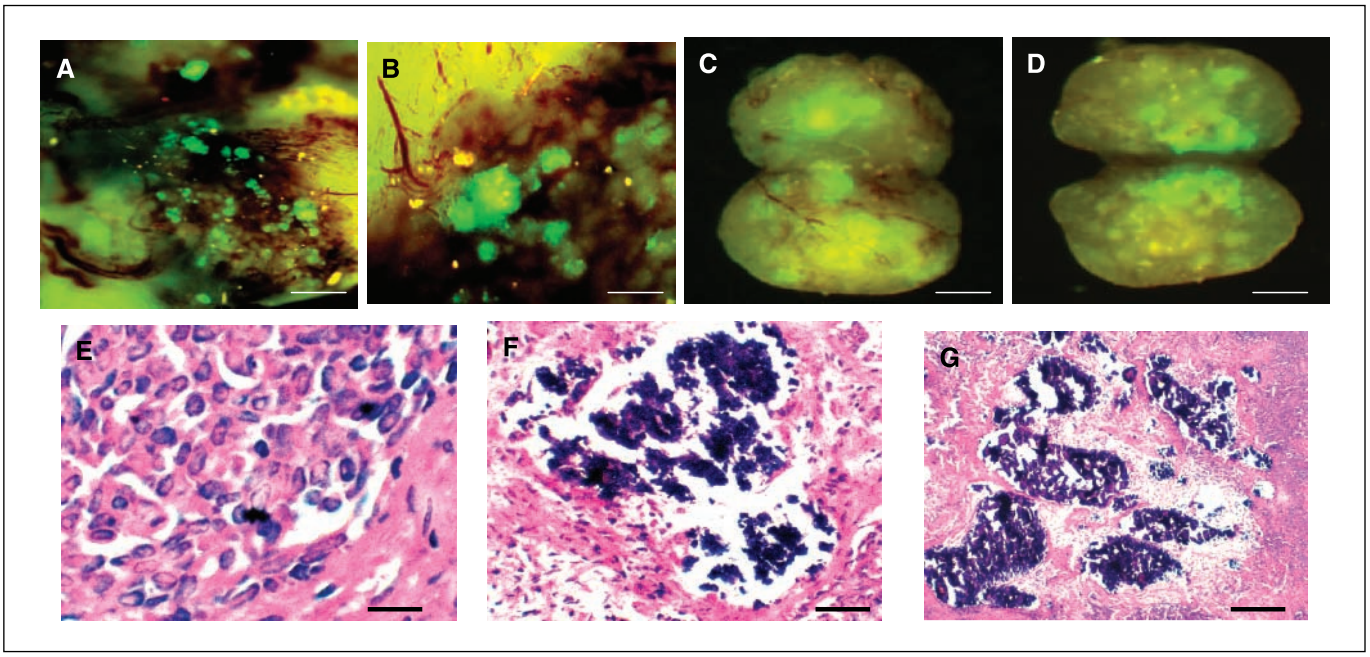

Figure 2. Growth of *S. typhimurium* A1-R in MARY-X human breast cancer tissue after tail vein injection. NCR nude mice, 6 to 8 weeks, were s.c. implanted in the midright side with human breast cancer tissue. Bacteria were injected directly into the tail vein (10^7 CFU/100 μL PBS). The tumors were excised, and samples were observed under fluorescence microscopy. For histology, the tumor tissue was fixed with 10% buffered formalin and processed for paraffin section and HE staining by standard methods. *A* and *B,* bacteria growing in tumor tissue on day 2 after infection. Bar, 150 μm in (*A* and *B*). *C,* surface image of bacterial distribution in intact tumor tissue on day 5 after infection. Bar, 180 μm. *D,* bacterial distribution in transverse section of tumor showing inner surface on day 5 after infection. Bar, 180 μm. *E,* bacteria growth in the viable MARY-X tumor tissue. Bar, 25 μm. *F,* bacteria destroyed tumor tissue (day 4 after infection). Bar, 50 μm. *G,* bacteria growth within the vascular regions of tumors (day 4 after infection). *Blue dots, arrows, S. typhimurium* A1-R. Bar, 100 μm.

Our approach to use a doubly auxotrophic leucine-arginine facultative anaerobic *S. typhimurium* strain has shown efficacy in prostate and breast tumors. Questions remain as to whether A1 or A1-R can target other tumor types. The selective approach, used in the present report, of passaging the bacteria through a tumor-bearing host increases the targeting capability of the bacteria. Passaging the bacteria through diverse types of tumors may therefore be a promising approach to expand the range of targets of the *S. typhimurium* amino acid auxotrophs. Auxotrophic mutations for other amino acids may be necessary for different types of tumors. These issues are important for the clinical development of tumor-targeting bacteria.

The ultimate goal of this research is to bring tumor-targeting bacterial therapy to the clinic. To achieve this goal, several issues need to be addressed. First and foremost is safety. Strains to be used in the clinic containing deletion mutations leading to the auxotrophy would have a lesser chance to revert to prototrophy than point mutants and therefore would be safer. It is essential that reversion to prototrophy does not occur, as this would lead to systemic infection of the patient. Therefore, future experiments will focus on deletion mutation–based auxotrophic tumor-targeting bacteria. Tumor-targeting bacteria to be used in the clinic would also have to have a broad range of efficacy against a particular type of tumor. The factors leading to a broad targeting range are still not well understood, and further work must be done to achieve this goal. Our results suggest that increased tumor cell adherence and invasion seem to be critical for increased tumor targeting by bacteria *in vivo*. The use of fluorescently tagged bacteria greatly facilitates tumor-targeting studies both *in vitro* and *in vivo*. Thus, fluorescently tagged bacteria may also be useful clinically. An important distinction of the present studies from previous approaches to bacterial therapy of cancer that used obligate anaerobes is that the facultative *S. typhimurium* strains used in this study can grow under aerobic conditions as well as anaerobic conditions. This property enables the bacteria to grow in the viable as well as in the necrotic area of the tumor resulting in greater efficacy. This ability enables the bacteria to be highly effective alone in preventing and reversing tumor growth. Thus, the future clinical use of these bacteria may not require toxic chemotherapy.

The following conclusions can be made from this study: (*a*) tumor cell adherence and invasion of *S. typhimurium* A1, auxotrophic for leucine and arginine, was enhanced by passage through a human colon cancer growing in mice following i.v.

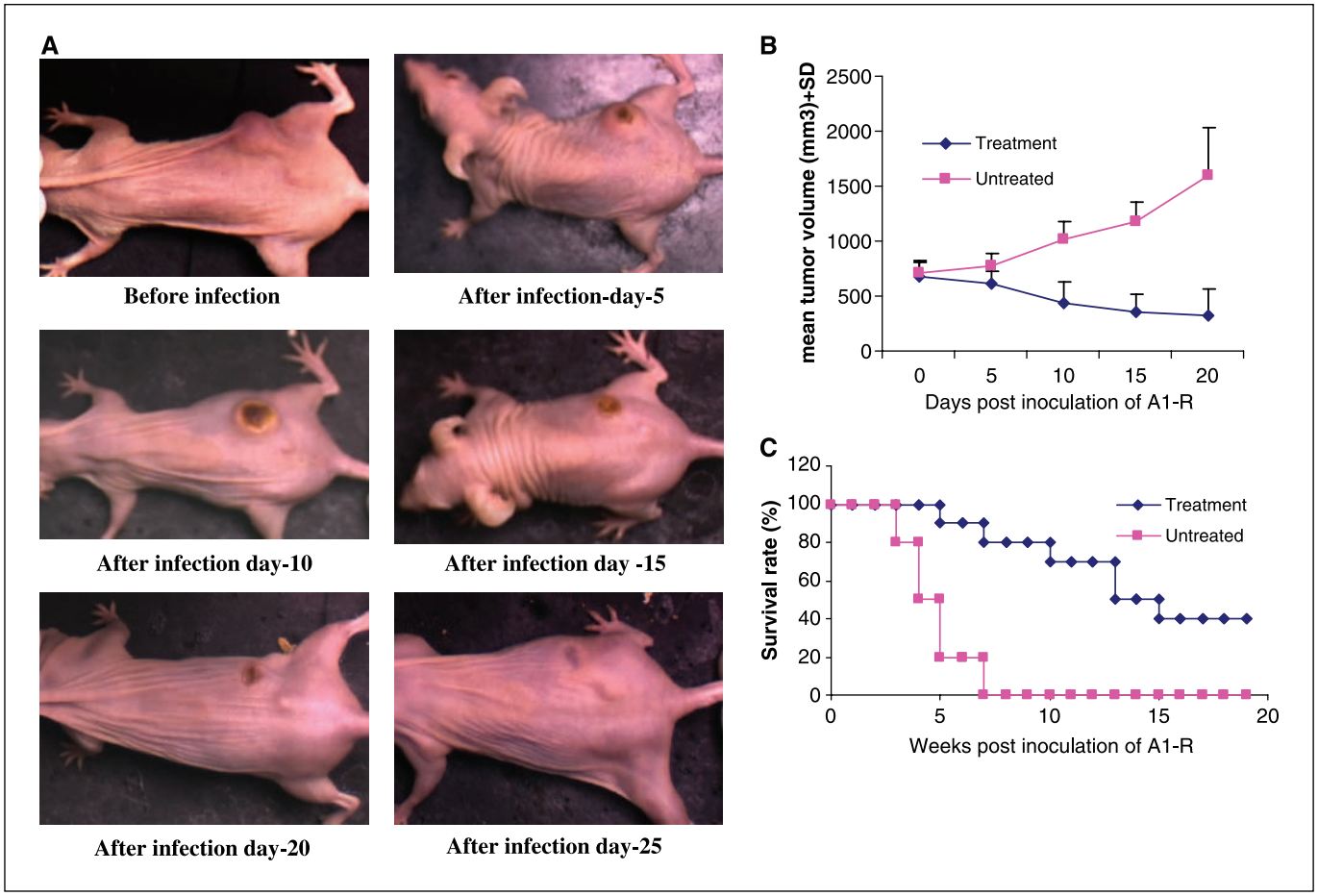

Figure 3. Efficacy of A1-R on MARY-X human breast cancer growing s.c. in nude mice. *A* and *B,* efficacy of A1-R on tumor growth. Six-week-old female *nu/nu* mice were s.c. implanted on the flank of the nude mice with 2 mm^3 MARY-X human breast cancer tissue. The bacteria were grown overnight on LB medium and then diluted 1:10 in LB medium. Bacteria were harvested in late-log phase, washed with PBS, and then diluted in PBS (10^7 CFU/100 μL PBS). *C,* efficacy of A1-R on survival of nude mice with MARY-X tumor. Tumor growth was monitored by whole-body imaging. Mice were sacrificed when the tumor mass reached 2 cm^3. Survival time was compared with the untreated mice. The survival of treated animals was prolonged, and the 40% cured mice survived as long as non–tumor-bearing mice. n = 10 mice for each group.

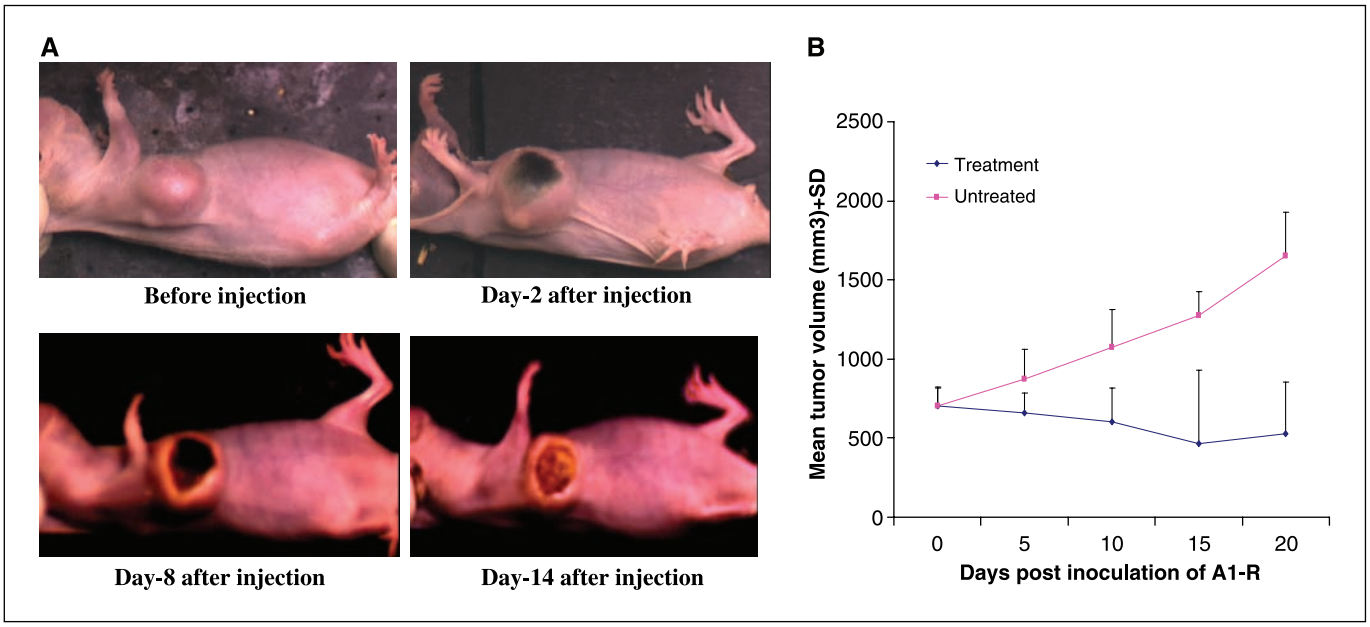

Figure 4. Efficacy of *S. typhimurium* A1-R on MARY-X human breast cancer growing orthotopically in nude mice. *A* and *B,* efficacy of A1-R on orthotopic MARY-X breast cancer. Nude mice, 6 to 8 weeks, were orthotopically implanted in the right second mammary gland fat pad with 2 mm^3 MARY-X human breast cancer tissue. A1-R bacteria were injected directly into the tail vein (10^7 CFU/100 μL PBS). Tumor size was measured at indicated time points after infection. n = 10 animals.

inoculation; (*b*) increased tumor cell adherence and invasion seem to be critical for increased bacteria tumor homing and growth in tumors; (*c*) the efficacy on tumor growth of *S. typhimurium* A1-R was enhanced by increased bacteria growth in the tumors; (*d*) nude mice implanted with human breast cancer MARY-X could be cured by *S. typhimurium* A1-R alone with a single i.v. injection. The tumor selectivity of A1 seems due to the arginine-leucine requirement as well as other mutations or variations resulting in increased tumor cell adherence, penetration, and intracellular growth; (*e*) the rich vascularity of the MARY-X tumor may also contribute to the bacterial targeting and efficacy; and (*f*) A1-R monotherapy could cure tumors as opposed to the need for combination therapy using anaerobic bacteria. This is probably due to the fact that A1-R targets viable tumor tissue.

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