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Efficacy of Tumor-Targeting *Salmonella Typhimurium* A1-R on Nude Mouse Models of Metastatic and Disseminated Human Ovarian Cancer

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ABSTRACT

We report here the efficacy of tumor-targeting *Salmonella typhimurium* A1-R (A1-R) on mouse models of disseminated and metastatic ovarian cancer. The proliferation-inhibitory efficacy of A1-R on human ovarian cancer cell lines (SKOV-3-GFP, OVCAR-3-RFP) was initially demonstrated in vitro. Orthotopic and dissemination mouse models of ovarian cancer were made with the human ovarian cancer cell line SKOV-3-GFP. After tumor implantation, the mice were treated with A1-R (5×10^7 colony-forming units [CFU], i.v.), and there were no severe adverse events observed. In the orthotopic model, tumor volume after treatment was $276 \pm 60.8\ mm^3$, compared to $930 \pm 342\ mm^3$ in the untreated control group ($P = 0.022$). There was also a significant difference in survival between treated mice and untreated mice in a peritoneal dissemination model ($P = 0.005$). The results of this report demonstrate that A1-R is effective for highly aggressive human ovarian cancer in metastatic and dissemination mouse models and suggest its clinical potential for this highly treatment-resistant disease. J. Cell. Biochem. 115: 1996–2003, 2014.

KEY WORDS: GFP; *Salmonella typhimurium*; BACTERIA; TUMOR-TARGETING; OVARIAN CANCER; NUDE MICE; IMAGING

Ovarian cancer is the fifth leading cause of cancer-related death among women in the U.S. Approximately 22,000 women receive a new diagnosis, and 14,000 die from ovarian cancer each year [Siegel et al., 2013].

For all types of ovarian cancer, the 5-year relative survival is 44%. If ovarian cancer is diagnosed before the cancer has spread outside the ovary, the 5-year relative survival rate is 92%. However, only 15% of all ovarian cancers are found at this early stage due to lack of symptoms and they tend to rapidly spread outside the ovary. If the cancer has distantly spread from the ovary, the 5-year survival rate is 27% [Howlader et al., 2012; American Cancer Society, 2013].

Cytoreductive surgery followed by platinum-based chemotherapy is first-line therapy for ovarian cancer with peritoneal dissemination [Cannistra et al., 2003]. However, most patients ultimately relapse with chemo-resistant disease. Angiogenesis inhibitors [O'Malley et al., 2011] and hyperthermic intraperitoneal chemotherapy (HIPEC) are used for advanced and recurrent disease [Bakrin et al., 2013; Cascales-Campos et al., 2013, 2014]. Other approaches, including viral therapy and molecular-targeting therapy, are being tested in dissemination mouse models of ovarian cancer [Takakura et al., 2010; Yang et al., 2011; Goshima et al., 2013].

Salmonella typhimurium, which is a facultative anaerobe, was previously attenuated with purine and other auxotrophic mutations and has been used for cancer therapy [Pawelek et al., 2003]. *S. typhimurium* with lipid A-modified (*msbB*) and purine auxotrophic (*purI*) mutations did not have toxicity in mice and swine and

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also had significantly reduced host TNF-α induction [Pawelek et al., 2003]. In a Phase I clinical trial on patients with metastatic melanoma and renal cell carcinoma, the *S. typhimurium* strain tested (VNP20009) was attenuated by *msbB* and *purI* mutations. VNP20009 was safely administered to patients but colonized the patients' tumors to a limited extent, perhaps because it was over-attenuated [Toso et al., 2002].

Another strain of *S. typhimurium*, A1-R, has been developed by our laboratory and has increased antitumor efficacy. *S. typhimurium* A1-R (A1-R) is auxotrophic for Leu-Arg which prevents it from mounting a continuous infection in normal tissues. A1-R has no other attenuating mutations as does VNP20009 and, therefore, may have higher tumor virulence. A1-R was able to eradicate primary and metastatic tumors in monotherapy in nude mouse models of prostate, breast, and pancreatic cancer, as well as sarcoma and glioma [Zhao et al., 2005, 2006, 2007; Hayashi et al., 2009; Nagakura et al., 2009; Kimura et al., 2010; Yam et al., 2010; Momiyama et al., 2012]. Tumors with a high degree of vascularity were more sensitive to A1-R, and vascular destruction appears to play a role in A1-R antitumor efficacy [Leschner et al., 2009; Liu et al., 2010].

S. typhimurium A1-R targeted the Lewis lung carcinoma (LLC) growing subcutaneously in nude mice whereby the bacterially infected cancer cells greatly expanded and burst and lost viability [Uchugonova et al., 2012].

We have developed a strategy to maximize efficacy and minimize toxicity for A1-R tumor-targeting in immunocompetent mice implanted with the LLC. A small primer dose of A1-R was first administered (1×10^6 colony-forming units [CFU] i.v.) followed by a high dose (1×10^7 CFU, i.v.) 4 h later. The primer-dose strategy resulted in smaller tumors and no observable side-effects compared to treatment with high-dose alone. Tumor vessel destruction was enhanced by primer dosing of A1-R in immunocompetent transgenic

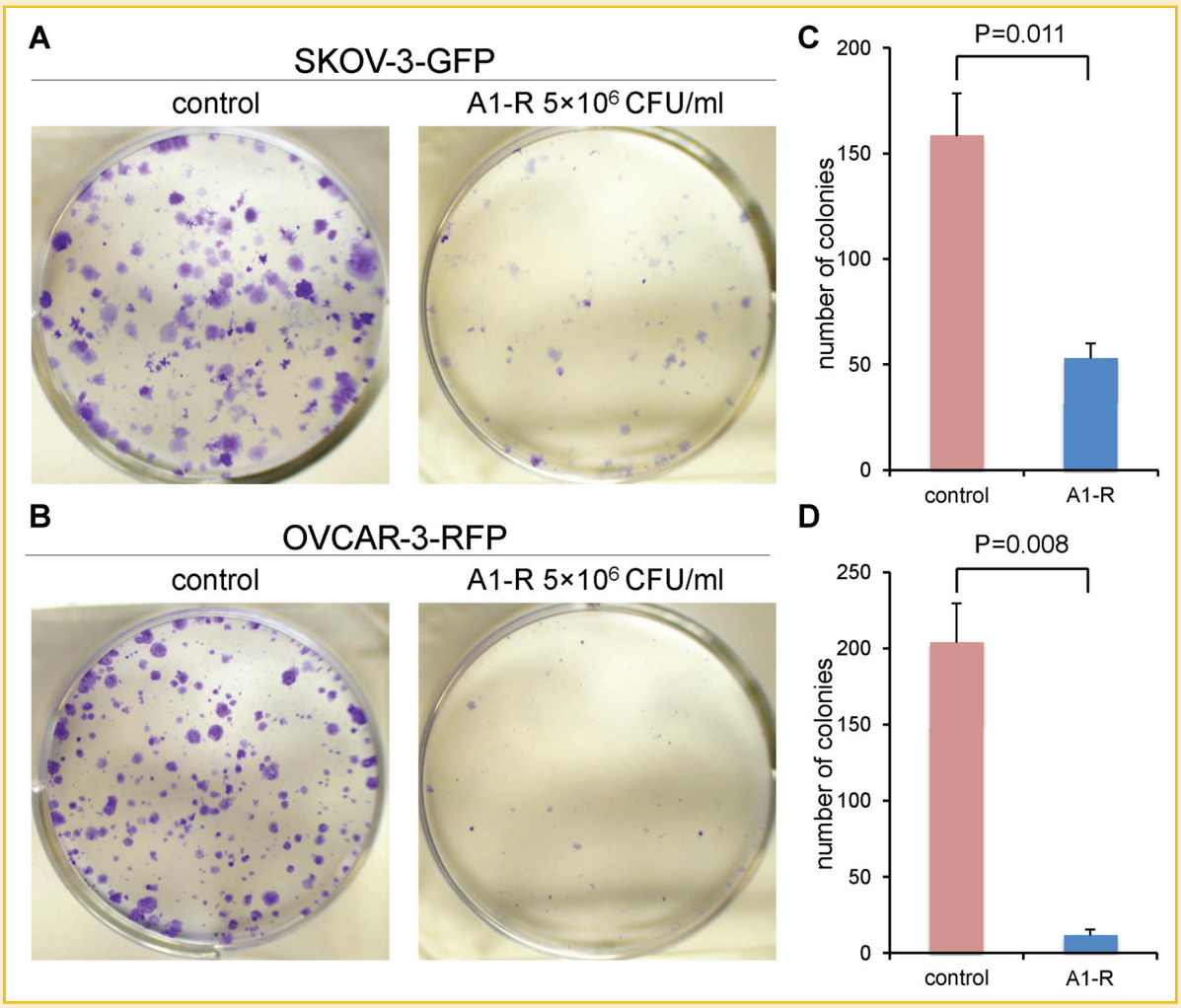

Fig. 1. Efficacy of *S. typhimurium* A1-R on ovarian cancer cell proliferation in vitro. A,B: Ovarian cancer cell death was induced by *S. typhimurium* A1-R in vitro. Ovarian cancer cells (A: SKOV-3-GFP, B: OVCAR-3-RFP) were grown in 6-well tissue culture plates to a density of approximately 5×10^2 cells/well. *S. typhimurium* A1-R GFP were grown to late log in LB broth, diluted in cell culture medium and added to the cancer cells with incubation at 37°C. After 60 min, the cells were rinsed and cultured in medium containing gentamycin sulfate (20 μg/ml) to kill external, but not internal, bacteria. After 7 days culture, the number of colonies was counted (Clonogenic Assay). C,D: Bar graphs show cancer-cell colony number (C: SKOV-3-GFP, D: OVCAR-3-RFP). There were significant differences between the untreated control groups and *S. typhimurium* A1-R-treated groups for each cell line ($P = 0.011$ for SKOV-3-GFP, $P = 0.008$ for OVCAR-3-RFP).

mice expressing the nestin-driven green fluorescent protein (ND-GFP), which is selectively expressed in nascent blood vessels [Tome et al., 2013].

We have also shown that A1-R can target chemo-resistant pancreatic cancer stem-like cells [Hiroshima et al., 2013] and pancreatic cancer patient-derived PDOX orthotopic xenographs [Hiroshima et al., 2014].

In the present study, we demonstrate that A1-R inhibits tumor growth, dissemination, and metastasis and extends survival in mouse models of aggressive human ovarian cancer.

MATERIALS AND METHODS

CELL LINES AND CULTURE CONDITIONS

SKOV-3-GFP [Buick et al., 1985] and OVCAR-3-RFP [Hamilton et al., 1983] human ovarian cancer cell lines (AntiCancer, Inc., San Diego, CA) were used for this study. Cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS). All media were supplemented with penicillin and streptomycin (Gibco-BRL). Cells were cultured at 37°C with 5% CO_2.

ANIMALS

Athymic nude mice (nu/nu) (AntiCancer, Inc.), 5–7 weeks, were used for this study. Mice were kept in a barrier facility under HEPA filtration. Mice were fed with autoclaved laboratory rodent diet. All animal studies were conducted in accordance with the principles and procedures outlined in the NIH guide for the Care and Use of Laboratory Animals under assurance number A3873-1.

GROWTH OF *S. TYPHIMURIUM* A1-R FOR TREATMENT

S. typhimurium A1-R GFP (AntiCancer, Inc.) [Zhao et al., 2006] were grown overnight in 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 ready for injection in mice.

EFFICACY OF *S. TYPHIMURIUM* A1-R ON OVARIAN CANCER CELL LINES

Human ovarian cancer cell lines (SKOV-3-GFP and OVCAR-3-RFP) were grown in 6-well plates to a density of 5×10^2 cells/ml. A1-R bacteria, expressing GFP, were grown in LB and added to the cancer cells (5×10^6 CFU/ml). After 60 min incubation at 37°C, the cells were rinsed and cultured in medium containing gentamycin sulfate (20 μg/ml) to kill external but not internal bacteria. After 7 days culture, the proliferation inhibitory efficacy of A1-R on the ovarian cancer cell lines was assessed colorometrically (Clonogenic Assay). ImageJ (National Institutes of Health, Bethesda, MD) was used to quantify the cell colonies (Fig. 1).

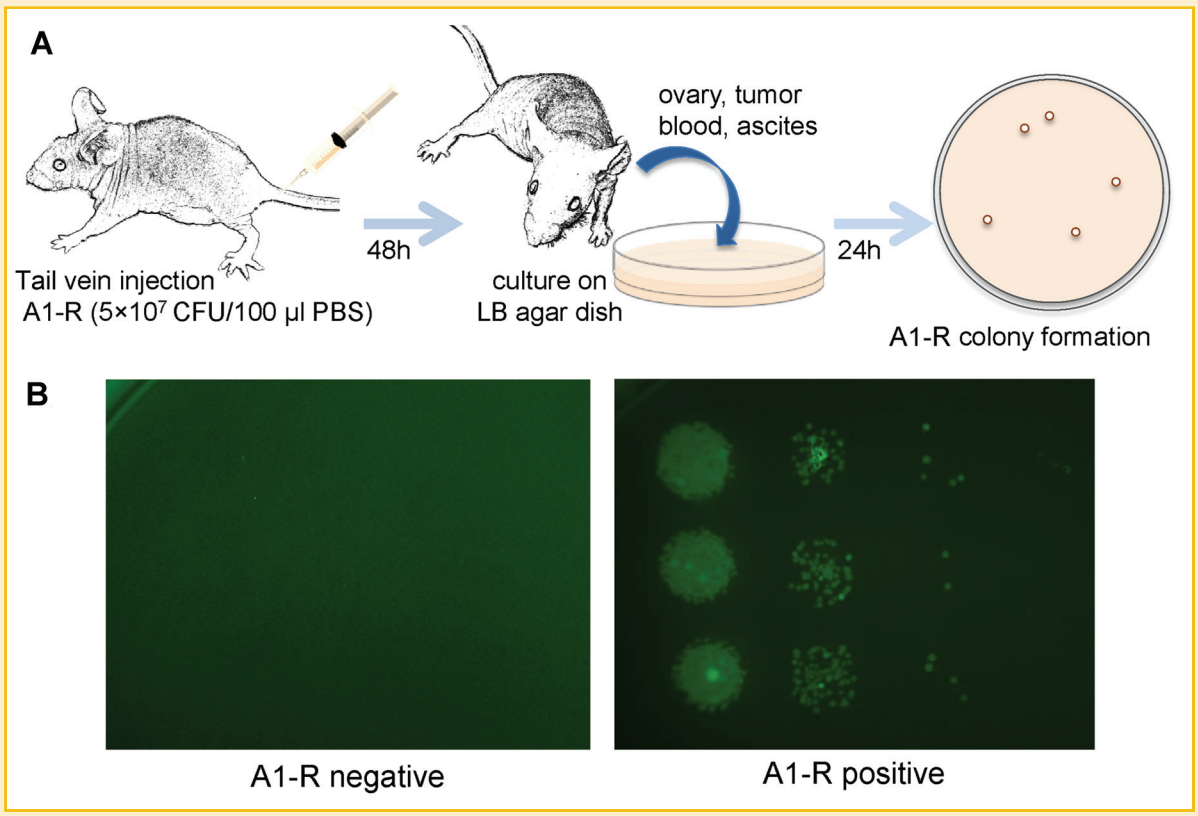

Fig. 2. Selectivity of *S. typhimurium* A1-R on ovarian cancer tumor targeting. A: *S. typhimurium* A1-R GFP (5×10^7 CFU, i.v.) was administered to four orthotopic tumor-bearing mice and four non-tumor bearing mice. Forty-eight hours after injection, the ovary, tumor, blood, and ascites were harvested and each tissue was cultured on LB Agar medium. The tissues were seeded in triplicate. Twenty-four hours after seeding, *S. typhimurium* A1-R-GFP colony formation were assessed using fluorescence imaging with the OV100 Small Animal Imaging System (Olympus, Tokyo, Japan) [Yamauchi et al., 2006]. B: Representative negative and positive colony formation (GFP) for ovary without and with tumor, respectively.

TUMOR TARGETING OF *S. TYPHIMURIUM* A1-R

To evaluate the affinity and specificity of tumor targeting, A1-R (5×10^6 CFU in 100 μl PBS, i.v.) was administered to four orthotopic tumor-bearing nude mice and four non-tumor-bearing nude mice. Forty-eight hours after injection, the blood, ascites, ovary with tumor (tumor bearing mice), and normal ovary (non-tumor bearing mice) were harvested. Each tissue was cultured on LB agar plates to determine the extent of infection with A1-R, which expressed GFP. After 24 h culture, colony formation was assessed by GFP fluorescence (Fig. 2).

SURGICAL ORTHOTOPIC IMPLANTATION (SOI) MODEL OF OVARIAN CANCER IN NUDE MICE

Subcutaneous tumors were first established by implantation of ovarian cancer cells (5×10^6–1×10^7 in 200 μl Matrigel) in the back skin of female nude mice (5–7 weeks), under anesthesia with a ketamine mixture (10 μl ketamine HCL, 7.6 μl xylazine, 2.4 μl acepromazine maleate, and 10 μl H_2O) administered by s.c. injection. For SOI, as previously described [Fu and Hoffman, 1993; Kiguchi et al., 1998], a right lateral dorsal incision was made, the retroperitoneum was opened, and one tumor block (2 mm × 2 mm) was implanted on the right ovarian capsule with an 8-0 surgical suture. The retroperitoneum and skin were closed with a 6-0 surgical suture (Fig. 3A).

EFFICACY OF *S. TYPHIMURIUM* A1-R ON THE OVARIAN CANCER SOI MODEL

Thirty days after SOI, eight mice were divided into two groups and tumor progression were assessed by whole-body GFP imaging. Four mice were treated with A1-R and the other four mice were untreated. A1-R (5×10^7 CFU, i.v.) was administered to the treatment group once a week for 3 weeks (30, 37, 44 days after implantation). All mice were necropsied 46 days after implantation for tumor removal and measurement in order to assess tumor growth (Fig. 3B). Fluorescence images were obtained with an Olympus OV100 Small Animal Imaging System (Olympus Corp, Tokyo, Japan). Intraperitoneal spread of tumor and distant metastasis were assessed at the same time. Tumor volume was calculated as $a \times b^2/2$ (a: major axis, b: minor axis).

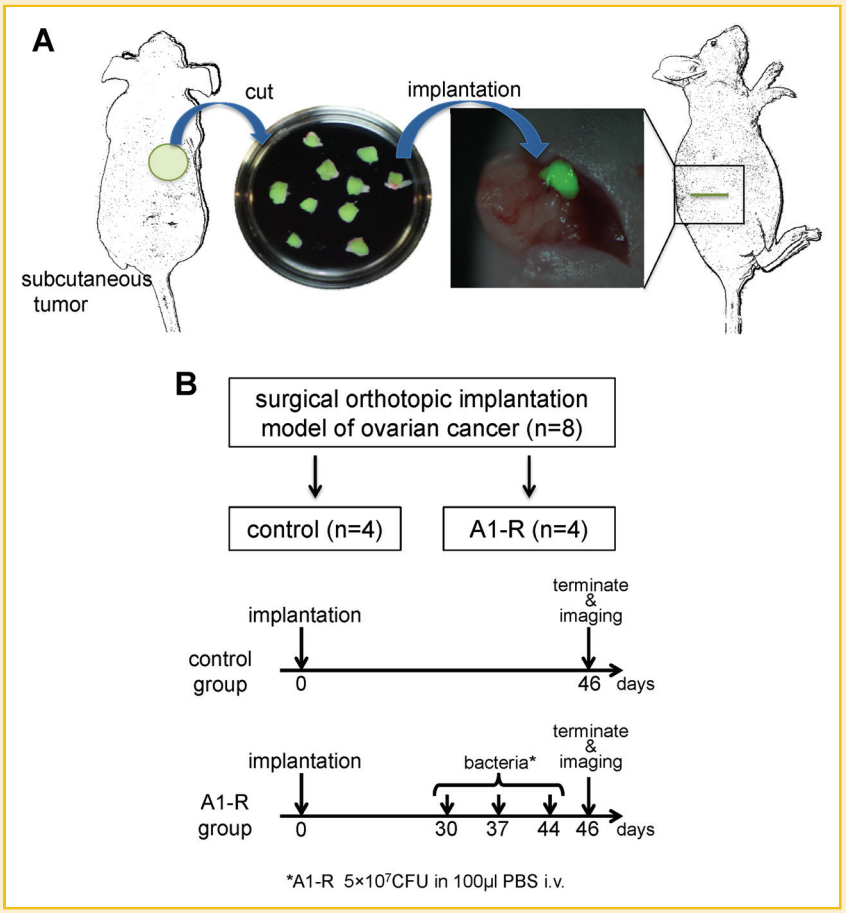

Fig. 3. Efficacy of *S. typhimurium* A1-R on orthotopic growth of human ovarian cancer in nude mice. A: Schematic of orthotopic model construction. SKOV-3-GFP subcutaneous tumors were harvested and cut into fragments of 2-mm diameter. A fragment of tumor was transplanted on the right ovary of nude mice using surgical orthotopic implantation (SOI) [Fu and Hoffman, 1993; Kiguchi et al., 1998]. B: Treatment schedule. Thirty days after implantation, tumors were measured via whole-body imaging with the OV100. Four mice were treated with *S. typhimurium* A1-R (5×10^7 CFU in 100 μl PBS, i.v.) on days 30, 37 and 44 and another four mice were used as untreated controls. On day 46, the tumors were exposed and imaged quantitatively to determine treatment efficacy.

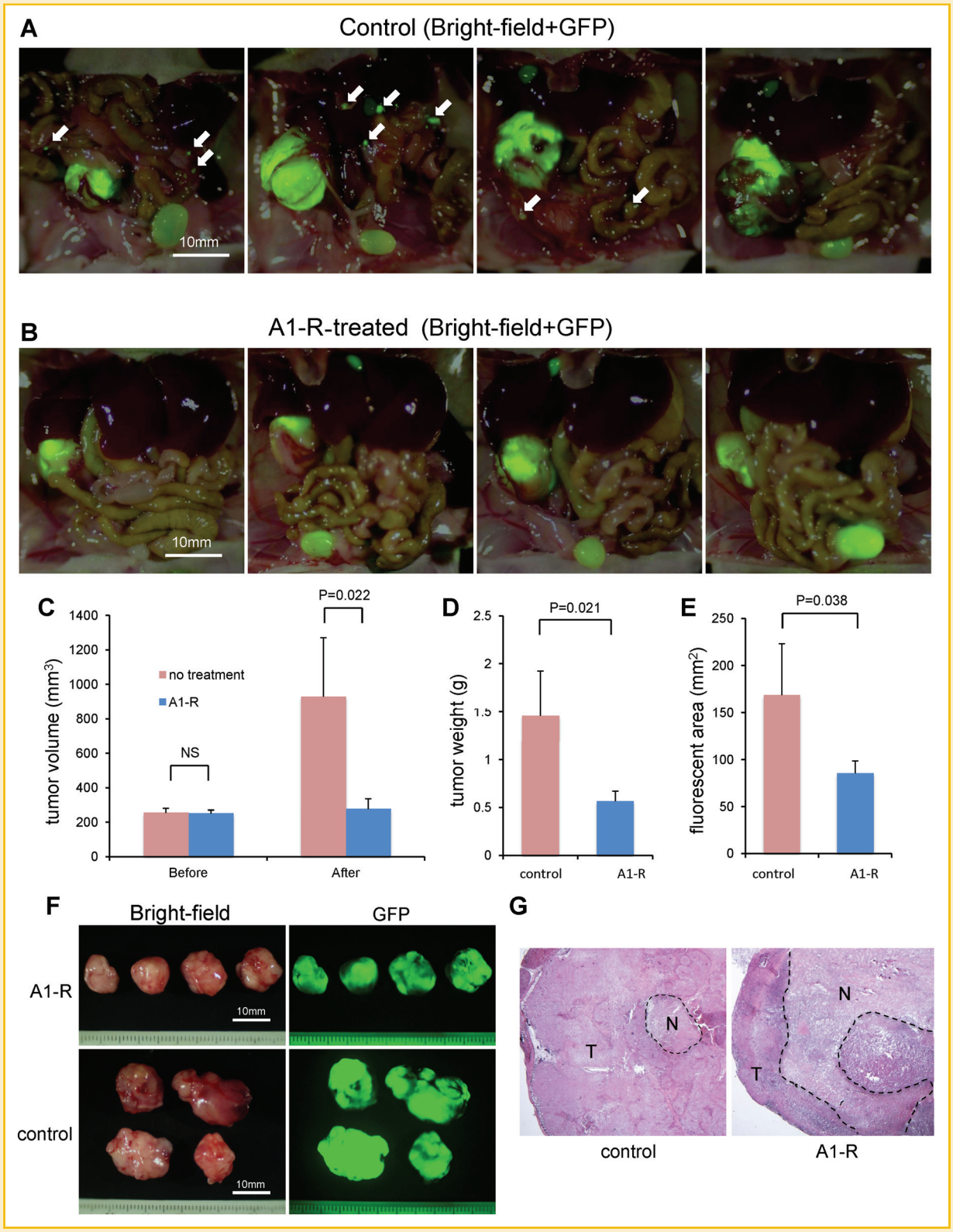

Fig. 4. Efficacy of *S. typhimurium* A1-R on orthotopic growth of human ovarian cancer in nude mice. A,B: Images of tumors at termination (A: control; B: *S. typhimurium* A1-R treated). Dissemination is seen in three of four untreated mice (white arrow). C: Treated and untreated mice had no difference in tumor volume before treatment began at 30 days after implantation. After treatment, there was a significant difference between the two groups in tumor volume (C, $P = 0.022$); tumor weight (D, $P = 0.021$); and tumor fluorescence area (E, $P = 0.038$). F: Bright-field and GFP imaging of primary tumors. G: Tumor histology, untreated and treated with *S. typhimurium* A1-R (H&E stain ×100). T = tumor with viable cells; N = necrosis. Dashed lines separate necrotic and viable tumor tissue.

DISSEMINATION MODEL OF OVARIAN CANCER IN NUDE MICE

A peritoneal dissemination mouse model was made with SKOV-3-GFP cells (5×10^6) which were injected into the peritoneal cavity of nude mice in 250 μl PBS. Formation of disseminated cancer foci in the peritoneal cavity was apparent by GFP imaging within 7 days after injection (Fig. 5A).

EFFICACY OF *S. TYPHIMURIUM* A1-R ON THE DISSEMINATION MODEL OF OVARIAN CANCER

Twenty mice were divided into two groups: one treated with A1-R and an untreated control group. A1-R (5×10^7 CFU, i.v.) was administered once every 7 days starting from 7 days after cell injection. Overall survival time of each group was assessed (Fig. 5B).

TOXICITY OF *S. TYPHIMURIUM* A1-R

The body weights of the study mice were measured weekly to assess toxicity of A1-R treatment.

STATISTICAL ANALYSIS

Data comparisons between two groups were assessed using the Student's *t*-test. The Kaplan–Meier method was used for survival determination, and the log-rank test was used for statistical significance of differences between the two groups. Differences were considered significant when $P < 0.05$. Data are expressed as mean ± standard deviation;1; (SD). Statistical analyses were performed with EZR (Saitama Medical Center, Jichi Medical University).

RESULTS AND DISCUSSION

CYTOTOXIC EFFICACY OF A1-R ON OVARIAN CANCER CELL LINES IN VITRO

Seven days after bacterial treatment, the number of SKOV-3-GFP colonies was 53.0 ± 7.0 in the A1-R-treated group compared to 158.3 ± 20.1 in the control group ($P = 0.011$; Figs. 1A and C). The number of OVCAR-3-RFP colonies was 11.7 ± 3.7 in the A1-R-treated group compared to 204 ± 25.7 in the control group ($P = 0.008$; Figs. 1B and D).

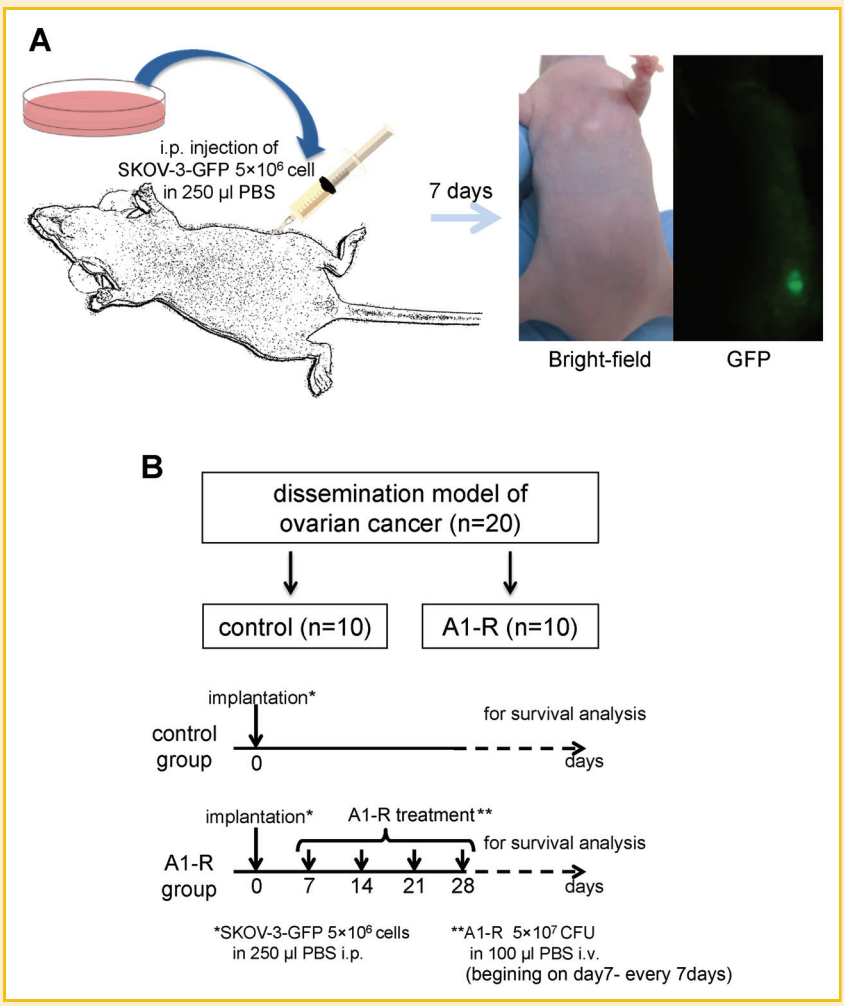

Fig. 5. Efficacy of *S. typhimurium* A1-R on dissemination of human ovarian cancer in nude mice. A: Schematic for constructing the dissemination model of ovarian cancer. SKOV-3-GFP (5×10^6 in 250 μl PBS) were injected in the intraperitoneal cavity. B: Treatment schedule. Seven days after implantation, tumor formation was confirmed with fluorescence imaging and mice were randomly divided into two groups. *S. typhimurium* A1-R (5×10^7 CFU i.v.) was administered every 7 days, starting from 7 days after implantation. Survival was determined 90 days after implantation.

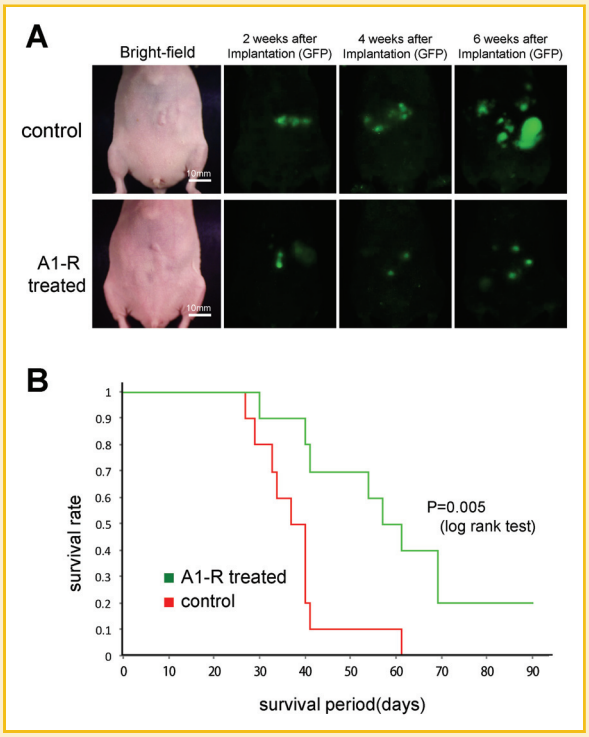

Fig. 6. Efficacy of *S. typhimurium* A1-R on dissemination of human ovarian cancer in nude mice. A: Representative time-course images of treated and control mice (bright-field and GFP imaging). B: Survival curve of treated and control groups of mice. There were significant differences between the two groups ($P = 0.005$, log-rank test).

TUMOR TARGETING *S. TYPHIMURIUM* A1-R

A1-R was administered to tumor-bearing and non-tumor-bearing nude mice. A1-R was detected in 75% (3/4) of ovaries with implanted tumor, 50% (2/4) of ascites, and 0% (0/4) in blood in tumor-bearing mice. A1-R was not detected in the ovary or blood of four non-tumor-bearing mice. These findings indicate A1-R was eliminated in normal mice and selectively targeted tumors (Fig. 2).

TUMOR SUPPRESSIVE EFFICACY OF *S. TYPHIMURIUM* A1-R IN THE SOI MODEL OF OVARIAN CANCER

Tumor size was $251 \pm 20.2\ mm^3$ in the group to be treated with A1-R and $255 \pm 26.1\ mm^3$ in the control group ($P = 0.84$) at the onset of treatment (Figs. 3 and 4). The sizes of the tumor ranged from 550 to $1{,}440\ mm^3$ in the control group and from 196 to $365\ mm^3$ in the treated group at the time of termination. There were significant differences in mean primary tumor volume ($276 \pm 60.8\ mm^3$ in the treated group compared to $930 \pm 342\ mm^3$ in the control group, $P = 0.022$). Mean tumor weight was 0.57 ± 0.11 g in the treated group compared to 1.46 ± 0.47 g in the control group ($P = 0.021$). Mean fluorescence area of the primary tumor was $85.7 \pm 12.9\ mm^2$ in the treated group compared to $168 \pm 54.7\ mm^2$ in the control group ($P = 0.038$; Figs. 3 and 4). Figures 4A and B shows the extent of local growth and peritoneal tumor spread in eight mice at the time of termination.

EFFICACY OF *S. TYPHIMURIUM* A1-R ON OVARIAN CANCER DISSEMINATION

Dissemination was observed in three of four control mice. In contrast, there was no dissemination in the *S. typhimurium* A1-R treated mice ($P = 0.028$, χ^2-test; Figs. 5 and 6). There was no metastasis to the liver or lung in either group.

EFFICACY OF *S. TYPHIMURIUM* A1-R ON SURVIVAL OF MICE WITH DISSEMINATED OVARIAN CANCER

To determine the survival efficacy of *S. typhimurium* A1-R on mice with disseminated ovarian cancer, A1-R treated and control mice were monitored for survival until 90 days after cancer cell implantation (Fig. 5). All untreated mice died between days 27 and 61, whereas *S. typhimurium* A1-R extended survival, with a 90% survival rate at day 40 and 20% survival rate on day 90 ($P = 0.005$, log rank test; Fig. 6).

The results of this report demonstrated that *S. typhimruium* A1-R has potential for treatment of advanced ovarian cancer. The combination of bacterial treatment and surgery or first-line chemotherapy will be evaluated in future studies on mouse models of advanced ovarian cancer.

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