

# Systemic Administration of Attenuated *Salmonella choleraesuis* in Combination with Cisplatin for Cancer Therapy

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Some anaerobic and facultative anaerobic bacteria have been employed as anticancer agents. Previously, we have demonstrated tumor-targeting and antitumor activities of attenuated *Salmonella choleraesuis* carrying antiangiogenic genes. Here we exploited *S. choleraesuis* as a single-agent therapy and as part of a combination therapy with low-dose cisplatin for syngeneic murine lung tumor and hepatoma. Systemically injected *S. choleraesuis* preferentially accumulated within tumors for at least 4 weeks and the bacteria accumulated preferentially in not only subcutaneous but also orthotopic tumors over livers and spleens at ratios ranging from 1000:1 to 100,000:1. *S. choleraesuis* was capable of delaying tumor growth and enhancing survival in both subcutaneous tumor and experimental metastasis models. More strikingly, the combination of *S. choleraesuis* plus cisplatin acted additively to retard tumor growth and extensively prolong the survival time of the mice bearing hepatomas or lung tumors. Such combination treatment also increased infiltrating neutrophils and CD8<sup>+</sup> T cells, as well as apoptotic cells, in the tumors, compared with *S. choleraesuis* or cisplatin treatment alone. These findings suggest that *S. choleraesuis* in combination with cisplatin, which exerts oncolytic effects and enhances antitumor immune responses, represents a promising strategy for the treatment of primary and metastatic tumors.

**Key Words:** *Salmonella choleraesuis*, cancer therapy, tumor targeted, cisplatin, combination therapy, hypoxia

## INTRODUCTION

The primary limitation of cancer therapy is lack of selectivity of therapeutic agents for tumor cells. Current efforts are focused on discovering and developing anti-cancer agents that selectively target only tumor cells and spare normal cells to improve the therapeutic index. The use of preferentially replicating bacteria as an oncolytic agent is one of the innovative approaches for the treatment of cancer. This is based on the observation that some obligate or facultative anaerobic bacteria are capable of multiplying selectively in tumors and inhibiting their growth. *Salmonella typhimurium*, a facultative anaerobe, has been employed as an antitumor agent that is capable of preferentially amplifying within tumors and inhibiting their growth [1–3]. Furthermore, these tumor-targeting bacteria have been used to deliver genes

encoding angiogenic inhibitors [4–6], prodrug-converting enzymes [7], or cytokines [8], aiming to enhance their oncolytic effects.

A hypoxic microenvironment is shared by many solid tumors. Hypoxia is also associated with a more malignant phenotype, which affects genomic stability, apoptosis, angiogenesis, and metastasis [9]. The hypoxic regions of tumors are less sensitive to ionizing radiation because its cell-killing effects depend on oxygen; they are also less sensitive to chemotherapeutic agents because drugs delivered to these regions may be suboptimal. The hypoxic areas of tumors are poorly vascularized and are not likely to be treated easily with conventional anti-cancer agents [10].

Since hypoxia is a common characteristic of human tumors, which adversely affects the prognosis of cancer patients, targeting the hypoxic regions of tumors may

increase the effectiveness of cancer treatment. We have used a vaccine strain of *Salmonella choleraesuis* as a live vector to carry DNA vaccines [11]. Recently, we demonstrated its tumor-targeting potential in various murine tumor models [4,5]. In this study, we exploited *S. choleraesuis* as a single tumor-targeting anticancer agent and as part of a combination therapy with chemotherapy for mice bearing syngeneic tumors. Our results indicate that the combination of *S. choleraesuis* and cisplatin exerts additive therapeutic effects in delaying tumor growth and prolonging survival of the tumor-bearing mice.

## RESULTS

### Tumor-Targeting Potential of *S. choleraesuis* in Immunocompetent Mice and Bacterial Colonization in the Hypoxic Regions of Tumors

We monitored the kinetics of the bacterial distribution after injection with  $2 \times 10^6$  colony-forming units (cfu) of *S. choleraesuis* into mice bearing syngeneic LL/2 lung tumors (Fig. 1A) or ML-1 hepatomas (Fig. 1B) implanted subcutaneously (s.c.). The bacterial amount was much higher in tumors than in livers and spleens in both strains of the mice at all the time points examined. Their amounts in the tumors continuously remained at a peak level for at least 4 weeks after bacterial inoculation and were approximately 3 to 5 orders of magnitude higher than those found in the livers or spleens. Whereas it was retained in the tumors at a constant level per gram of tumor, we detected *S. choleraesuis* to a much lesser extent in the spleens or livers at day 28. Notably, as tumors became larger at later time points examined, total

bacterial number within the tumor actually increased with time, indicating that the bacteria not only preferentially accumulated but also amplified within the tumors. By contrast, as early as day 7, *S. choleraesuis* was undetectable in the blood from all the mice tested (data not shown). Determination of the LD<sub>50</sub> of the attenuated *S. choleraesuis* after intraperitoneal administration in mice revealed that its virulence was reduced by approximately  $10^6$ -fold, compared with that of a local virulent *S. choleraesuis* strain, Dadin, isolated from swine.

To examine whether *S. choleraesuis* targeted the hypoxic regions in tumors, we injected *S. choleraesuis* into LL/2 tumor-bearing mice and observed the bacterial distribution and hypoxic regions within tumors. As shown in Fig. 1C, *S. choleraesuis* predominantly resided, not absolutely but very closely, in the hypoxic regions of the tumor, whereas it was scarcely detected in the spleen that was not hypoxic. In addition to being hypoxic, tumor microenvironment is very likely to be immunosuppressive, while the spleen is significantly more immunologically active. Therefore, the immunosuppressive microenvironment around the tumor may also contribute, in part, to the preferential accumulation of *S. choleraesuis* in tumors.

### Bacterial Accumulation and Antitumor Effects Observed in both Treated Ipsilateral and Untreated Contralateral Tumors Following Intratumoral Injection of *S. choleraesuis*

We also examined whether *S. choleraesuis* could target the untreated tumor when injected intratumorally (i.t.) into one of the bilaterally implanted LL/2 tumors.

**FIG. 1.** Preferential accumulation of *S. choleraesuis* (S.C.) in the tumors and its colonization in the hypoxic regions of tumors from mice administered systemically with *S. choleraesuis*. Mice bearing (A) LL/2 or (B) ML-1 tumors were injected i.p. with  $2 \times 10^6$  cfu of *S. choleraesuis* at day 0. The amounts of accumulated *S. choleraesuis* in the tumors, livers, and spleens were determined at days 7, 14, 21, and 28. Each value represents the mean  $\pm$  SD from five mice. (C) Detection of *S. choleraesuis* in the hypoxic regions of tumors. LL/2 tumor-bearing mice were injected i.p. with *S. choleraesuis* ( $2 \times 10^6$  cfu), and at 28 days p.i. tumors and spleens were removed for immunofluorescence double staining with anti-Hypoxyprobe-1 antibody to visualize hypoxic regions by Texas red and with anti-*S. choleraesuis* serum to visualize the bacteria by fluorescein. Nuclei were counterstained with DAPI. The Merge column represents the superposition of the tumor sections stained with anti-Hypoxyprobe-1, anti-*S. choleraesuis*, and DAPI to visualize colocalization. Spleen was used as a negative control for hypoxic staining. Localizations of hypoxic regions and *S. choleraesuis* were observed under fluorescence microscope at an original magnification of  $\times 200$ .

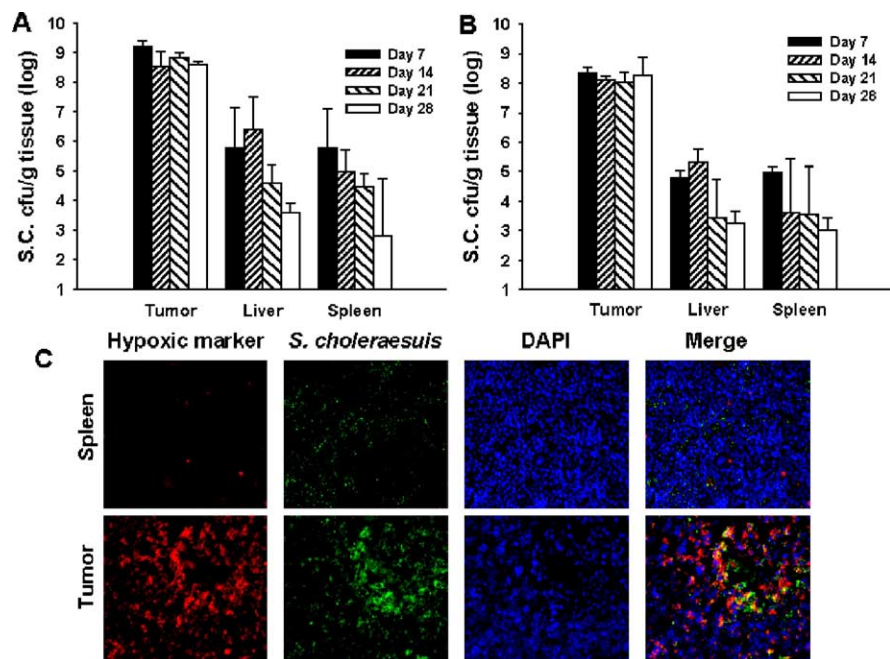


Fig. 2A shows that *S. choleraesuis*, when injected i.t. into the left tumor, preferentially accumulated not only in the treated tumors but also in the untreated contralateral tumors at 7 days postinfection (p.i.), although the bacterial number in the treated tumor was slightly higher than that in the untreated one. The growth of both tumors ipsilateral (Fig. 2B) and contralateral (Fig. 2C) to the bacteria injection site was significantly retarded in the *S. choleraesuis*-treated mice compared with that in PBS-treated mice. We also noted preferential accumulation of *S. choleraesuis* in bilaterally implanted tumors after intraperitoneal (i.p.) injection of the bacteria (Fig. 2D), resulting in retarded bilateral tumors (Figs. 2E and 2F). Taken together, these results indicate that *S. choleraesuis*, administered via either intratumoral or systemic route, was able to accumulate in the tumors at distant sites and, as a consequence, conferred contralateral antitumor effect. Thus, this tumor-targeting property of *S. choleraesuis* provides the impetus to explore its use in inhibiting tumor growth at distant sites.

#### Preferential Accumulation of *S. choleraesuis* in Orthotopic Tumors

To ensure that the tumor-targeting potential of *S. choleraesuis* was not confined to the subcutaneous site, but rather was a general phenomenon, we investigated its tumor-targeting potential in more clinically relevant cancer models, namely the ML-1

model of orthotopic hepatoma and the highly aggressive LL/2 model of experimental metastasis. We inoculated mice bearing ML-1 liver nodules or LL/2 lung nodules, and normal healthy mice, i.p. with *S. choleraesuis* and determined the amounts of *S. choleraesuis* in the tumors, livers, and spleens after bacterial inoculation. We found a notable amount of *S. choleraesuis* in the orthotopic hepatoma, whereas *S. choleraesuis* was detected to a much lesser extent in the spleens or healthy livers (Fig. 3A). As shown in Fig. 3B, although there was no significant difference between tumor-bearing and normal healthy mice in the amount of *S. choleraesuis* that accumulated in the livers and spleens, where no tumors were found, the bacterial number per gram tissue of the lungs with LL/2 nodules was significantly higher than that from normal healthy mice. Taken together, *S. choleraesuis* accumulated in not only subcutaneous but also orthotopic tumors after systemic administration.

#### Reduction of Metastatic Nodules and Prolongation of Survival Time of the Mice Bearing Experimental Metastasis by Systemic Delivery of *S. choleraesuis*

Because inhibition of metastatic tumor growth is still a major challenge for cancer treatment, we next investigated whether *S. choleraesuis* could inhibit established pulmonary tumor nodules. We treated mice with the bacteria 15 days after establishment of pulmonary nodules and monitored their growth 5 days later. We

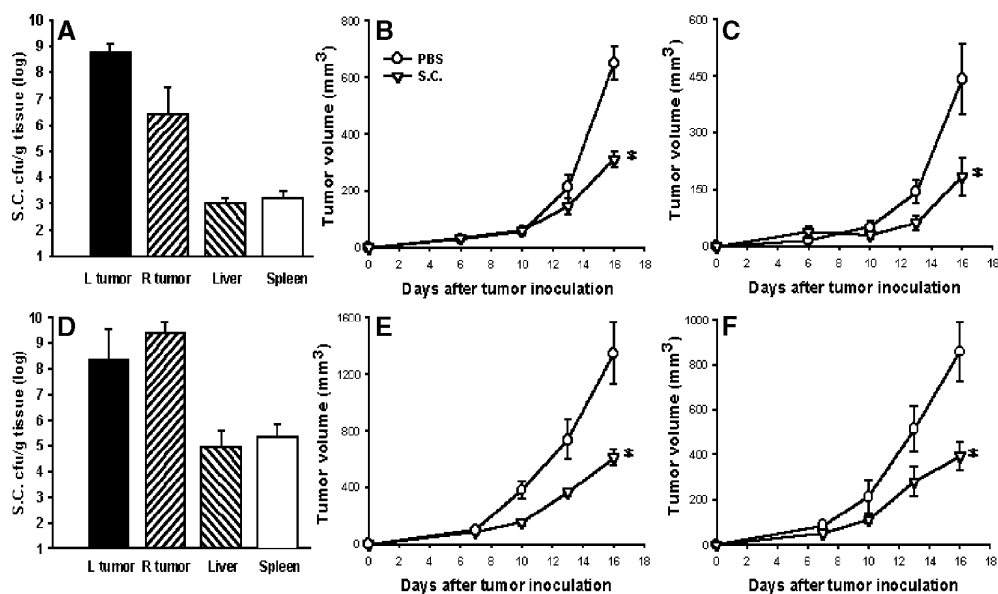
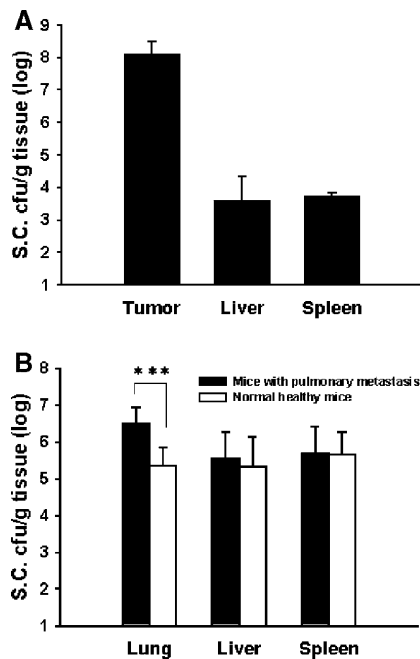


FIG. 2. Preferential accumulation of *S. choleraesuis* (S.C.) in the bilateral tumors from mice administered locally or systemically with *S. choleraesuis*. *S. choleraesuis* ( $2 \times 10^6$  cfu) or PBS was injected i.t. into the left flank tumor or i.p. to C57BL/6 mice bearing bilateral LL/2 tumors at day 8. The amounts of accumulated *S. choleraesuis* (means  $\pm$  SD,  $n = 4$ ) in the left (L) and right (R) flank tumors, livers, and spleens of mice injected (A) i.t. or (D) i.p. with the bacteria were determined at day 15. Differences in the tumor volumes (means  $\pm$  SEM,  $n = 4$ ) of the (B, E) left and (C, F) right flank tumors between the mice treated with *S. choleraesuis* and PBS were compared at day 16. \* $P < 0.05$ .



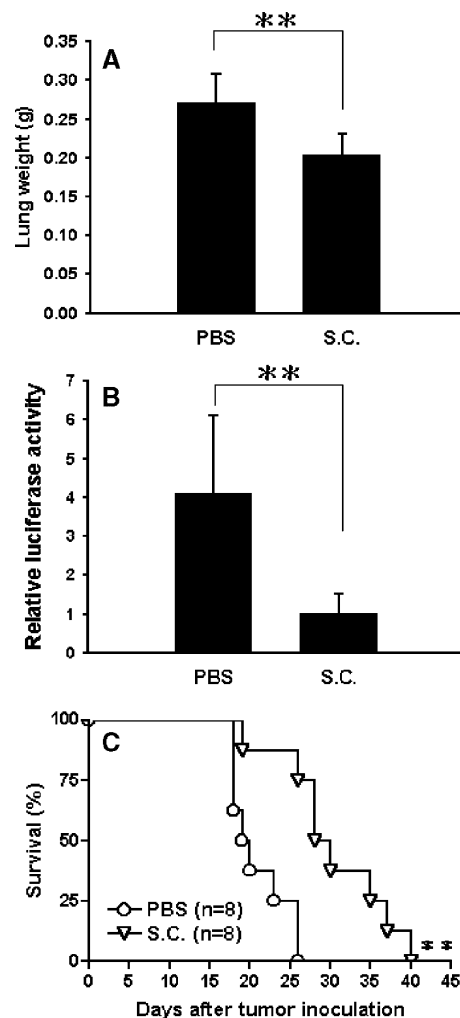
**FIG. 3.** Preferential accumulation of *S. choleraesuis* (S.C.) in the orthotopic tumors from mice administered systemically with *S. choleraesuis*. (A) BALB/c mice bearing orthotopic ML-1 tumors were injected i.p. with *S. choleraesuis* ( $2 \times 10^6$  cfu) at day 18. The amounts of accumulated *S. choleraesuis* (means  $\pm$  SD,  $n = 4$ ) in the tumors, livers, and spleens were determined at day 38 ( $P < 0.001$  for tumor versus liver or spleen). (B) C57BL/6 mice that had been injected with LL/2 cells ( $10^5$ ) via the tail vein at day 0 were injected i.p. with *S. choleraesuis* ( $2 \times 10^6$  cfu) at day 15. A parallel experiment was performed using normal healthy mice injected with *S. choleraesuis*. The amounts of *S. choleraesuis* accumulated in the lungs, livers, and spleens from mice with pulmonary metastasis (means  $\pm$  SD,  $n = 8$ ) and normal healthy mice (means  $\pm$  SD,  $n = 7$ ) were determined at day 20. \*\*\* $P < 0.001$ .

found that tumor nodules with various sizes were disseminated and overlapped, making the quantification of tumor nodules difficult. To facilitate quantification of the metastatic tumor burden in the lungs, we induced pulmonary metastasis by using LL/2-Luc cells expressing luciferase. We injected mice with LL/2-Luc cells via the tail vein at day 0, treated them i.p. with *S. choleraesuis* or PBS at day 15, and killed them at day 20. A majority of the lungs from PBS-treated mice possessed numerous confluent pulmonary nodules, whereas lungs from the mice treated with *S. choleraesuis* exhibited smaller and fewer tumor nodules. To quantify further the tumor burden, we measured the wet lung weight and luciferase activity. The mice treated with *S. choleraesuis* had 24% less wet lung weight compared with those treated with PBS (Fig. 4A). Moreover, the level of luciferase expression in the lungs from *S. choleraesuis*-treated mice was approximately 75% less than that from PBS-treated mice (Fig. 4B). *S. choleraesuis* treatment also significantly prolonged the survival time of the mice with pulmonary metastasis compared with their PBS-treated counterparts

(Fig. 4C). Collectively, these results indicate that systemic delivery of *S. choleraesuis* delayed tumor growth in the lungs and enhanced survival of the mice bearing pulmonary metastatic tumors.

#### Additive Antitumor Effects of the Combination Therapy of *S. choleraesuis* and Cisplatin

Although *S. choleraesuis* monotherapy was effective in retarding the growth of established lung cancer, complete tumor regression was not observed. We, therefore, determined whether it was more effective if administered in conjunction with chemotherapy. We chose to use cisplatin because this compound has been frequently



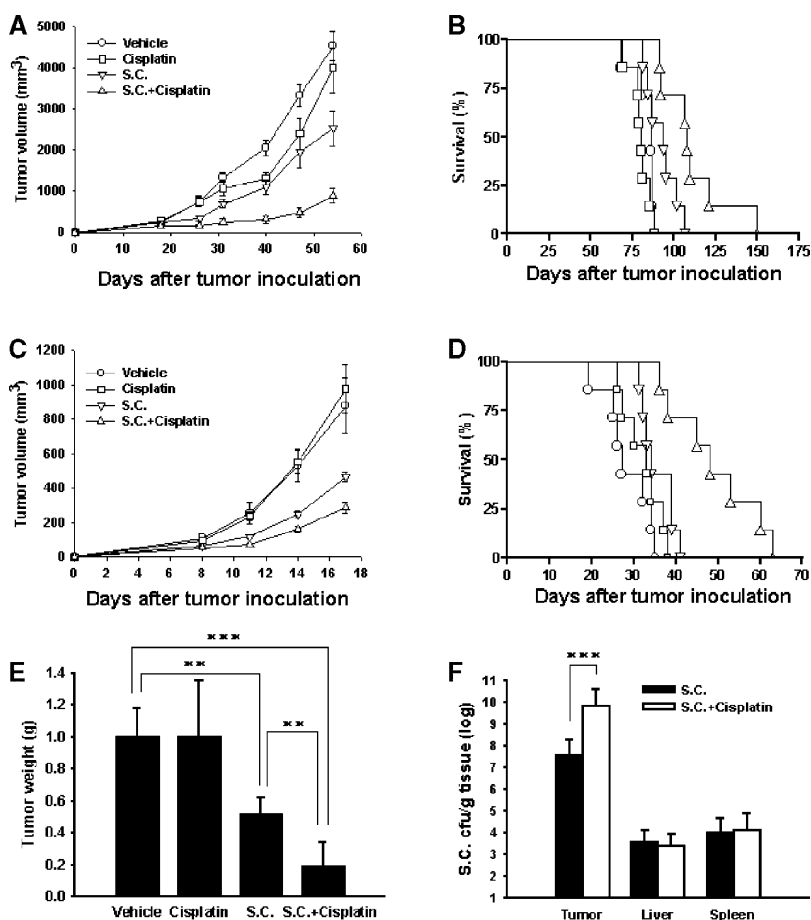
**FIG. 4.** Antitumor effects of *S. choleraesuis* (S.C.) on mice bearing pulmonary metastatic tumors. Groups of eight C57BL/6 mice that had been inoculated with  $10^5$  (A, B) LL/2-Luc or (C) LL/2 cells via the tail vein at day 0 were treated i.p. with *S. choleraesuis* ( $2 \times 10^6$  cfu) at day 15. (A) The wet lung weight and (B) luciferase activity of the lungs were measured at day 20. Each value represents the mean  $\pm$  SD ( $n = 8$ ). (C) Kaplan-Meier survival curves at day 40 are shown. \*\* $P < 0.01$ .

used in the clinical treatment of hepatoma and lung cancer. We evaluated the antitumor effects of *S. choleraesuis* alone or combined with low-dose cisplatin in terms of tumor growth and survival of the mice bearing ML-1 or LL/2 tumors. In mice bearing either ML-1 or LL/2 tumors, treatment with *S. choleraesuis* alone significantly retarded tumor growth (Figs. 5A and 5C) and prolonged the survival time (Figs. 5B and 5D) compared with vehicle treatment. While treatment with cisplatin given in three doses exerted no antitumor effect, the combination treatment of *S. choleraesuis* plus cisplatin significantly retarded tumor growth and enhanced survival compared with *S. choleraesuis* treatment alone (Figs. 5A, 5B, 5C, and 5D). The mean tumor volume of ML-1 tumor-bearing mice treated with *S. choleraesuis* plus cisplatin was lowered by 55, 77, and 80% compared with those treated with *S. choleraesuis*, cisplatin, and PBS alone, respectively (Fig. 5A). Similarly, the mean tumor volume of LL/2 tumor-bearing mice treated with *S. choleraesuis* plus cisplatin was lowered by 46, 70, and 67% compared with those treated with *S. choleraesuis*, cisplatin, and PBS alone, respectively (Fig. 5C). Taken together, *S. choleraesuis* as a single-agent therapy could retard tumor growth

and enhance survival in both the fast-growing LL/2 and the slow-growing ML-1 tumor models. More strikingly, additive antitumor effects could be achieved with the combination therapy of *S. choleraesuis* plus cisplatin. In accordance with the results shown in Fig. 5C, the tumor weight from LL/2 tumor-bearing mice treated with *S. choleraesuis* plus cisplatin was 62, 80, and 81% lower than that of those treated with *S. choleraesuis*, cisplatin, and PBS alone, respectively (Fig. 5E). Accordingly, the amount of *S. choleraesuis* (cfu/g tissue) in the tumors from mice treated with *S. choleraesuis* in combination with cisplatin was approximately 100-fold higher than that from mice treated with *S. choleraesuis* alone (Fig. 5F).

### Increased Infiltrating Neutrophils and CD8<sup>+</sup> T Cells, as Well as Apoptotic Cells, in the Tumors Following the Combination Treatment of *S. choleraesuis* and Cisplatin

We analyzed the tumors from LL/2-bearing mice treated with *S. choleraesuis* or cisplatin alone, or in combination, for cell infiltrates by immunohistochemical staining and for apoptotic cells by the terminal deoxynucleotidyl-transferase-mediated deoxyuridine triphosphate nick-end



**FIG. 5.** Additive antitumor effects of *S. choleraesuis* (S.C.) in combination with cisplatin on subcutaneous ML-1 and LL/2 tumors. (A, B) Groups of eight BALB/c mice that had been inoculated s.c. with ML-1 cells ( $10^6$ ) at day 0 were treated i.p. with *S. choleraesuis* ( $2 \times 10^6$  cfu) at day 18 followed by cisplatin (2 mg/kg) at days 25, 27, and 29 or with either treatment alone. (C, D, E, F) Groups of eight C57BL/6 mice that had been inoculated s.c. with LL/2 cells ( $10^6$ ) at day 0 were treated i.p. with *S. choleraesuis* ( $2 \times 10^6$  cfu) at day 8 followed by cisplatin (2 mg/kg) at days 15, 17, and 19 or with either treatment alone. Vehicle control mice received PBS. Tumor volumes (means  $\pm$  SEM,  $n = 8$ ) among different treatment groups were compared in mice bearing (A) ML-1 ( $P < 0.001$  for S.C. + cisplatin versus cisplatin or vehicle;  $P < 0.01$  for S.C. + cisplatin versus S.C. and for S.C. versus vehicle) and (C) LL/2 tumors ( $P < 0.001$  for S.C. + cisplatin versus cisplatin;  $P < 0.01$  for S.C. + cisplatin versus S.C. or vehicle;  $P < 0.05$  for S.C. versus vehicle). Kaplan–Meier survival curves of the mice bearing (B) ML-1 ( $P < 0.001$  for S.C. + cisplatin versus cisplatin or vehicle;  $P < 0.05$  for S.C. + cisplatin versus S.C. and for S.C. versus vehicle) and (D) LL/2 tumors ( $P < 0.01$  for S.C. + cisplatin versus S.C., cisplatin, or vehicle;  $P < 0.05$  for S.C. versus vehicle) with different treatments are shown. In another set of experiments in the LL-2 tumor model, (E) the tumor weight and (F) the amounts of *S. choleraesuis* accumulated in the tumors, livers, and spleens (means  $\pm$  SD,  $n = 8$ ) were measured at day 20. \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ .

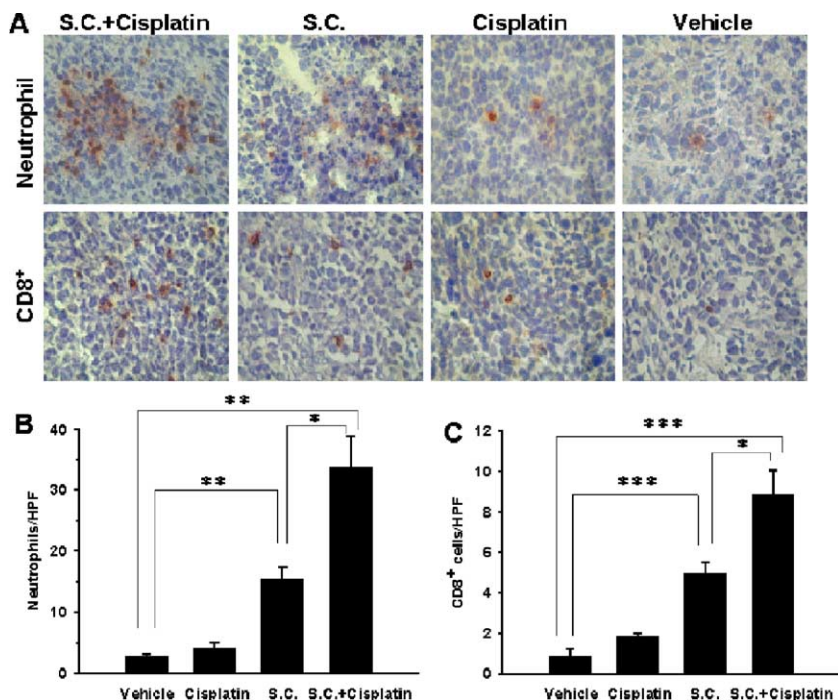
labeling (TUNEL) assay. Representative results for immunohistochemistry are shown in Fig. 6A. We observed notable increases of neutrophils and CD8<sup>+</sup> T cells that infiltrated the tumors in the mice treated with *S. choleraesuis* and, in particular, in those treated with *S. choleraesuis* plus cisplatin. The numbers of infiltrating neutrophils and CD8<sup>+</sup> T cells in the tumors treated with *S. choleraesuis* plus cisplatin were significantly increased compared with those in the tumors from the remaining three treatment groups, whereas no such difference was found between cisplatin- and PBS-treated groups (Figs. 6B and 6C). However, the levels of infiltrating CD4<sup>+</sup> T cells, which were in a very small amount, were similar among the different treatment groups (data not shown). TUNEL assay showed an increase in the amount of cells undergoing apoptosis in the *S. choleraesuis*-treated tumors compared with cisplatin-treated or PBS-treated tumors (Figs. 7A and 7B). Notably, cisplatin alone failed to promote tumor apoptosis. Nevertheless, there was a twofold increase in the number of apoptotic cells induced by *S. choleraesuis* plus cisplatin compared with that induced by *S. choleraesuis* alone (Fig. 7B). Of interest is the finding that the mice treated with cisplatin alone, compared with those treated with PBS, revealed reduced intratumoral microvessel density (mean  $\pm$  SEM 20.92  $\pm$  1.42/high-power field versus 26.92  $\pm$  1.51/high-power field,  $n = 4$ ;  $P < 0.05$ ). Taken together, these results indicate that the combination therapy with *S. choleraesuis* and low-dose cisplatin resulted in retarded tumor growth and an increase in infiltrating neutrophils and CD8<sup>+</sup> T

cells, as well as enhanced apoptosis in the tumors. Furthermore, the antiangiogenic effect exerted by low-dose cisplatin may have contributed, at least in part, to the enhanced antitumor effect of the combination regimen.

## DISCUSSION

Bacteria have been exploited as an antitumor agent because they can be motile and grow in hypoxic areas of tumors [1]. Not only attenuated bacterial strains, such as *S. typhimurium* [1–3], *Vibrio cholerae*, and *Listeria monocytogenes*, but also wild-type bacteria, such as *Escherichia coli* DH5 $\alpha$  strain, exhibit tumor-targeting potential, indicating that no mutations affecting the survival of the bacteria are required for tumor-targeting potential [12]. Previously, we exploited an attenuated *S. choleraesuis* as a delivery vehicle for DNA vaccine [11] and to carry eukaryotic expression vectors encoding antiangiogenic molecules as an anticancer agent [4,5]. This rough variant of *S. choleraesuis*, designated vaccine 51, was obtained by spreading an 18-h broth culture of the virulent strain 188 of *S. choleraesuis* subsp. *choleraesuis* serovar Dublin over the surface of a dried nutrient agar plate and placing a drop of a suspension of *Salmonella* anti-O phage 1 and selecting for a phage-resistant colony after incubation at 37°C for 24 h [13]. In this study, we confirm and extend the findings regarding the usefulness of *S. choleraesuis* as a tumor-targeted anticancer agent. Our results demonstrate the capability of *S. choleraesuis* to target and multiply in

**FIG. 6.** Increases in neutrophil and CD8<sup>+</sup> T cell infiltrates in the tumors from LL/2 tumor-bearing mice treated with *S. choleraesuis* (S.C.) in combination with cisplatin. Groups of four C57BL/6 mice that had been inoculated s.c. with LL/2 cells ( $10^6$ ) at day 0 were treated i.p. with *S. choleraesuis* ( $2 \times 10^6$  cfu) at day 8 followed by cisplatin (2 mg/kg) at days 15, 17, and 19 or with either treatment alone. Vehicle control mice received PBS. (A) Tumors were excised at day 20 and immunostained with antibodies against Gr-1 or CD8<sup>+</sup> (original magnification  $\times 400$ ). (B) Neutrophils and (C) CD8<sup>+</sup> T cells that infiltrated tumors were determined by averaging the cell numbers from three fields of the highest positive-stained cell density at  $\times 400$  magnification in each section (means  $\pm$  SEM,  $n = 4$ ). \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ .



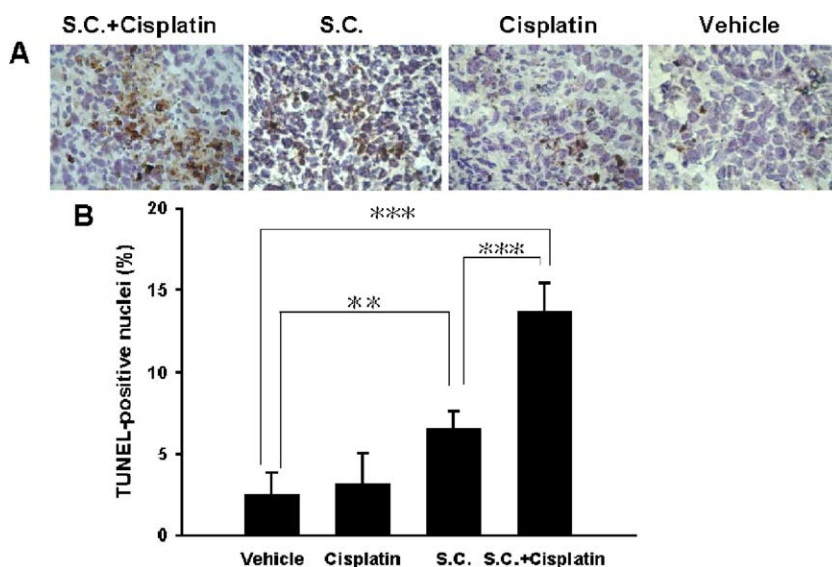


FIG. 7. Increase in tumor cells undergoing apoptosis in LL/2 tumor-bearing mice treated with *S. choleraesuis* (S.C.) in combination with cisplatin. Groups of four C57BL/6 mice that had been inoculated s.c. with LL/2 cells ( $10^6$ ) at day 0 were treated i.p. with *S. choleraesuis* ( $2 \times 10^6$  cfu) at day 8 followed by cisplatin (2 mg/kg) at days 15, 17, and 19 or with either treatment alone. Vehicle control mice received PBS. (A) Tumors were excised at day 20, and TUNEL assay was used to detect apoptotic cells (original magnification  $\times 400$ ). (B) TUNEL-positive cells were counted from three fields of the highest density of positive-stained cells in each section to determine the percentage of apoptotic cells (means  $\pm$  SEM,  $n = 4$ ). \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ .

primary tumors at both subcutaneous and orthotopic sites via systemic administration. The bacteria preferentially accumulated and amplified within implanted tumors in mice for at least 4 weeks, whereas in our previous studies *S. choleraesuis* carrying expression plasmids were retained within tumors for a shorter period of time [4,5]. Because bacteria containing plasmids that confer antibiotic resistance have lower growth rates and tend to lose plasmids gradually *in vivo*, they appear to accumulate at lower levels and reside for a shorter period of time within tumors than bacteria without plasmids. The results shown in the present study, together with our previous findings [4], demonstrate that *S. choleraesuis* predominantly resides in the hypoxic regions of tumors. Furthermore, by comparing the tumor-targeting ability of *S. choleraesuis* in immunocompetent and immunodeficient mice, we have shown previously that although the degrees of initial colonization and early replication of the bacteria within tumors are equivalent regardless of the immune status of the host, around 1 week, when adaptive immune responses are elicited, the bacteria are cleared more rapidly not only from the spleen and liver but also from the tumor of immunocompetent mice [4]. The bacteria may enter tumors through leaky vasculature, thereby escaping the immune surveillance of the host and finding sanctuary within tumor tissues [12]. Therefore, *Salmonella* may be cleared more rapidly from livers and spleens, where abundant immune cells are present, than from tumors by the host immune surveillance, thereby persisting longer within tumors. In addition, hypoxic regions within tumors, where most necrotic regions are found, may provide more nutrients that selectively favor the growth of *Salmonella*.

In the work described here, we employed LL/2 lung tumor models of bilaterally implanted subcutaneous lung

tumors and experimental metastatic nodules to investigate the tumor-targeting potential of *S. choleraesuis* via intratumoral or systemic administration. Our results show that intratumoral injection of *S. choleraesuis* not only replicated within the treated tumor but also spread to and accumulated in the untreated contralateral tumor at distant sites. Furthermore, *S. choleraesuis* accumulated and replicated not only in subcutaneous LL/2 tumors, but also in metastatic small nodules after systemic administration. More importantly, we also demonstrated preferential accumulation of *S. choleraesuis* in a more clinically relevant orthotopic hepatoma model in immunocompetent mice. Therefore, the ability of *S. choleraesuis* to target multiple tumors from distant sites makes it an ideal anticancer agent over some other cancer therapeutic agents limited to local administration. The propensity of *S. choleraesuis* to accumulate and amplify within primary tumors and metastatic nodules may have contributed to retarding tumor growth and metastasis, as well as prolonging the survival of tumor-bearing mice. Recently, we showed the therapeutic efficacy of *S. choleraesuis* carrying eukaryotic expression vectors encoding antiangiogenic molecules, namely endostatin or thrombospondin-1, which exhibits a dual effect involving tumoricidal and antiangiogenic activities on tumor growth, for the treatment of syngeneic murine bladder tumor and melanoma [4,5]. In the present study, we demonstrate that the combination of *S. choleraesuis* with low-dose cisplatin induced an additive effect on retarding tumor growth and prolonging the survival time in the highly aggressive LL/2 lung tumor and slowly growing ML-1 hepatoma models.

In the present study, despite the failure of cisplatin to exhibit antitumor effects, its combination with *S. choleraesuis* enhanced the antitumor efficacy of *S.*

*choleraesuis* and promoted accumulation of the bacteria within tumors in the murine lung tumor model. Notably, while cisplatin administered alone neither increased cell infiltrates nor induced apoptosis in the tumors, *S. choleraesuis* treatment alone significantly increased infiltrating neutrophils and CD8<sup>+</sup> T cells as well as apoptotic cells within tumors, compared to vehicle treatment. Surprisingly, the combination treatment of *S. choleraesuis* and cisplatin resulted in a further twofold increase in cell infiltrates and apoptosis in the tumors. Tumors from the mice treated with cisplatin alone revealed a significant decrease in microvessel density compared with those from the control mice. However, this activity appeared insufficient to inhibit tumor growth or enhance survival of LL/2 tumor-bearing mice. In the context of tumor angiogenesis, cisplatin may have pleiotropic activities that can affect angiogenic processes directly and indirectly. In this regard, previous reports have documented that cisplatin in combination with some antiangiogenic agents decreases angiogenesis and increases apoptosis in tumors, resulting in enhanced antitumor effects in murine tumor models, compared with either treatment alone [14–16]. To this end, some low-dose chemotherapeutic agents also exhibit antiangiogenic activities, which are mediated by a systemic increase in thrombospondin-1 levels [17,18]. In addition to its antiangiogenic effect, cisplatin enhances expression of the cell death receptor, Fas, in various cancer cells, including LL/2 lung tumor cells. This effect may, in turn, augment cytotoxic T-cell-mediated antitumor immunity [19]. In this study, immunohistochemical studies reveal that combined therapy with *S. choleraesuis* plus cisplatin or *S. choleraesuis* monotherapy increased neutrophils and CD8<sup>+</sup> T cells that infiltrated tumors. The persistence of high levels of *Salmonella* within tumors may have induced inflammatory responses, leading to the recruitment of immune cells, such as macrophages, neutrophils, and lymphocytes to the tumor site. Since bacterial replication in tumors and subsequent lysis of tumor cells may induce cell-mediated immune responses to tumor cells, higher oncolysis could account, in part, for an increased infiltrate of CD8<sup>+</sup> T cells in *S. choleraesuis*-treated tumors. The cytotoxic T cell response against tumor cells may enhance the antitumor efficacy of *S. choleraesuis*. Antitumor effects of neutrophils, in particular, after being activated by substances derived from microorganisms, have also been demonstrated in various tumors [20–22]. Moreover, lipopolysaccharide from *Salmonella* may induce apoptosis of tumor and endothelial cells [23]. The potentiation of antiangiogenesis and upregulation of cytotoxic T cell responses by cisplatin may contribute to the enhanced antitumor effect of *S. choleraesuis* in conjunction with cisplatin.

In this study, we exploited a vaccine strain of *S. choleraesuis* as a tumor-targeted anticancer agent in

murine syngeneic tumor models. However, the mutations responsible for attenuation in this vaccine strain have not been genetically defined [13]. VNP20009, an attenuated strain of *S. typhimurium* [24], and its derivative, TAPET-CD, which expresses an *E. coli* cytosine deaminase (CD) [7], have been evaluated as anticancer agents in clinical trials for cancer patients [25,26]. VNP20009 is modified from a wild-type strain of *S. typhimurium* by partial deletion of the *msbB* gene, which results in the generation of a lipid A mutant with diminished ability to induce TNF- $\alpha$ -mediated septic shock [3]. It is also a purine auxotroph and cannot metabolize xylose. Recently, the targeting and replication of VNP2009 expressing herpes simplex virus thymidine kinase in murine tumors *in vivo* have been noninvasively detected by positron emission tomography imaging [27]. It has been shown that *S. typhimurium* has limited ability to adhere to tumor vasculature and migrate within tumors and survives only in tissue that becomes necrotic [28]. As *S. typhimurium* accumulates in necrotic regions of tumors that are distant from tumor vasculature, systemic delivery of the prodrug 5-fluorocytosine to the necrotic regions of tumors may be suboptimal in tumor-bearing mice treated with *S. typhimurium* expressing CD, which may contribute to the failure of the treatment in eradicating tumors. Furthermore, *S. typhimurium* has been demonstrated to grow in the necrotic and relatively hypoxic foci within tumors, but not in well-oxygenated tumors at the rim of the growing nodules [29]. The limited ability of *S. typhimurium* to disperse throughout the tumor may be the most important shortcoming in its use as an anticancer agent [29]. Regarding the importance of the dispersal capability of bacteria for tumoricidal activity, Dang *et al.* [30] showed that *Clostridium novyi*, an obligate anaerobe, is able to disperse evenly and eradicate tumors in mice when combined with an antivascular agent. Therefore, a genetically modified *Salmonella* strain capable of dispersing more homogeneously throughout tumors would be a more desirable tumoricidal agent or transgene delivery vector. Our previous results suggest that production of antiangiogenic agents via *S. choleraesuis*-mediated gene transfer increases *S. choleraesuis* accumulation in tumors and, as a consequence, enhances tumoricidal effects. In this study, our data suggest that the capability of *S. choleraesuis* to disperse within tumors and hence to delay tumor growth was augmented when combined with low-dose cisplatin. An additional strategy to enhance antitumor efficacy would involve inhibiting the viable rim of tumor growth or modifying the tumor matrix that would facilitate the motility and penetration of *Salmonella*.

By taking advantage of the tumoricidal effect of *Salmonella* and pleiotropic activities of cisplatin, we conclude that *S. choleraesuis* in combination with cisplatin appears to hold promise for the treatment of solid tumors. However, further work is warranted to elucidate

the underlying mechanism of antitumor effects of the combination therapy of *S. choleraesuis* and cisplatin.

## MATERIALS AND METHODS

**Bacteria, cell lines, and mice.** A vaccine strain of *S. choleraesuis* [*S. choleraesuis* subsp. *choleraesuis* (Smith) Weldin serovar Dublin (ATCC 15480)] was obtained from the Bioresources Collection and Research Center (Hsinchu, Taiwan) [13]. The LL/2 mouse Lewis lung carcinoma cell line was obtained from the American Type Culture Collection. The ML-1 mouse hepatoma cell line was originally established from hepatocytes of BALB/c mice [31]. LL/2 and ML-1 cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 1% glutamine, and 50 µg/ml gentamicin at 37°C in 5% CO<sub>2</sub>. LL/2 cells cultured in six-well plates were transfected with 2 µg of pTCYLuc [4], and clonal derivatives were isolated by G418 (400 µg/ml) selection and expanded to independent LL/2-Luc clones. Male C57BL/6 and BALB/c mice (6–8 weeks of age) were obtained from the Laboratory Animal Center of the National Cheng Kung University. The experimental protocol adhered to the rules of the Animal Protection Act of Taiwan and was approved by the Laboratory Animal Care and Use Committee of the National Cheng Kung University.

**Assay of tumor-targeting potential of *S. choleraesuis* and detection of hypoxic areas within tumors.** C57BL/6 and BALB/c mice were inoculated s.c. with 10<sup>6</sup> LL/2 and ML-1 cells, respectively. When the tumors had grown to 100 to 450 mm<sup>3</sup>, which took around 8 days for LL/2 tumor and 18 days for ML-1 tumor, the mice were injected i.t. or i.p. with 2 × 10<sup>6</sup> cfu of *S. choleraesuis*. At various time points p.i., five mice in each group were sacrificed, and the numbers of *S. choleraesuis* in the tumors, livers, and spleens were determined on LB agar plates and expressed as cfu per gram of tissues as previously described [4]. Meanwhile, at 28 days p.i., we examined the locations of *S. choleraesuis* and hypoxic regions within LL/2 tumors as described [4]. In the bilateral tumor model, LL/2 cells (10<sup>6</sup>) were inoculated s.c. into both flanks of C57BL/6 mice at day 0. Subsequently, *S. choleraesuis* (2 × 10<sup>6</sup> cfu) or PBS was injected i.t. into the left flank or i.p. into the mice at day 8. The amounts of accumulated *S. choleraesuis* in the left and right tumors, livers, and spleens of four mice in each group were determined at day 15. In the orthotopic ML-1 tumor model, groups of four BALB/c mice were anesthetized with 90 mg/kg ketamine hydrochloride, their abdominal cavities opened, and liver lobes exposed, and ML-1 cells (5 × 10<sup>5</sup>) were injected into the left liver lobe at day 0 using a 10-µl syringe (Terumo, Elkton, MD, USA). Notably, the needle hole was sealed with an electric coagulator immediately after withdrawal of the needle to avoid leakage of the injected fluid. The incision was subsequently sutured. In the murine model of experimental pulmonary metastasis, groups of eight C57BL/6 mice were injected with LL/2 cells (10<sup>5</sup>) via the tail vein at day 0. Subsequently, *S. choleraesuis* (2 × 10<sup>6</sup> cfu) was injected i.p. into the ML-1 tumor-bearing mice at day 18 or into the LL/2 tumor-bearing mice at day 15, and *S. choleraesuis* that accumulated in the tumors or lungs, as well as livers and spleens, were quantified at day 38 (for ML-1 tumor) or day 20 (for LL/2 tumor). A parallel experiment was performed using normal healthy mice inoculated with *S. choleraesuis*, and the bacteria in the respective organs were also assessed.

**Treatment of primary lung tumor and experimental pulmonary metastasis with *S. choleraesuis*.** *S. choleraesuis* (2 × 10<sup>6</sup> cfu) or PBS was injected i.t. into the left flank tumor or i.p. into groups of four C57BL/6 mice bearing bilateral LL/2 tumors. Palpable tumors in both sides were measured as described previously [5]. To enhance the ability to quantify tumor burden in the lungs, pulmonary metastasis was induced by inoculation of LL/2-Luc cells (10<sup>5</sup>) via the tail vein into C57BL/6 mice to evaluate the antimetastatic effect of *S. choleraesuis*. The bacteria (2 × 10<sup>6</sup> cfu) or PBS was then inoculated i.p. into groups of eight mice on day 15, and the wet lung weight and luciferase activity of the lungs were measured at day 20 [5]. In another set of experiments, LL/2 cells rather than LL/2-Luc cells were used to induce pulmonary metastasis and the

same treatment regimen was applied. Survival of the mice in the treated and control groups was monitored daily.

**Treatment of subcutaneous lung tumor and hepatoma with *S. choleraesuis* in combination with low-dose cisplatin.** We used subcutaneous LL/2 and ML-1 tumor models to evaluate the antitumor efficacy of *S. choleraesuis* (2 × 10<sup>6</sup> cfu) in combination with low-dose cisplatin. C57BL/6 and BALB/c mice were inoculated s.c. with 10<sup>6</sup> LL/2 or ML-1 cells at day 0, and visible nodules developed at all injection sites with approximate tumor volumes of 100 and 450 mm<sup>3</sup> at days 8 and 18, respectively. Groups of eight LL/2 tumor-bearing mice were injected i.p. with *S. choleraesuis* on day 8 followed by cisplatin (2 mg/kg) treatment on days 15, 17, and 19 or with either treatment alone. Similarly, groups of eight ML-1 tumor-bearing mice were treated with *S. choleraesuis* on day 18 followed by cisplatin treatment on days 25, 27, and 29 or with either treatment alone. All of the mice were monitored for tumor growth and survival as previously described [5].

**Immunohistochemistry and apoptotic assays.** To analyze cell infiltrates in the tumors, groups of four C57BL/6 mice that had been inoculated s.c. with 10<sup>6</sup> LL/2 cells at day 0 were injected i.p. with either 2 × 10<sup>6</sup> cfu of *S. choleraesuis* at day 8, or low-dose cisplatin (2 mg/kg) at days 15, 17, and 19, or both. Control mice received PBS. The tumors were excised and snap frozen at day 20. Cryostat sections (4 µm) were prepared, fixed, and incubated with rat anti-mouse Ly-6G (Gr-1) (RB6-8C5; PharMingen, San Diego, CA, USA), rat anti-mouse CD4 (L3T4) (GK1.5; PharMingen), or rat anti-mouse CD8a (Ly-2) (53-6.7; PharMingen) antibody. After sequential incubation with appropriate peroxidase-labeled secondary antibody and aminoethyl carbazole as substrate chromagen, the slides were counterstained with hematoxylin. The infiltrating cells were quantified by averaging the number of each cell type in three areas of highest cell density at ×400 magnification in each section. Tumor vascularization was determined by counting factor VIII-positive vessels with immunohistochemical analysis, and microvessel density was determined by averaging the number of vessels in three areas of highest vessel density at ×400 magnification in each section [5]. TUNEL assay was used to detect cell apoptosis within tumors and was performed according to the manufacturer's instructions (Promega, Madison, WI, USA). TUNEL-positive cells (brown staining) were counted under the microscope. We counted three high-power (×400) fields with approximately 200–250 cells that showed the highest density of positive-stained cells per field to determine the average percentage of apoptotic (TUNEL-positive) cells in each section.

**Statistical analysis.** The unpaired, two-tailed Student *t* test was used to determine differences between groups for the comparisons of tumor volume, tumor weight, lung weight, luciferase activity, and numbers of *S. choleraesuis*, neutrophils, CD8<sup>+</sup> T cells, factor VIII-positive cells, and apoptotic cells. The survival analysis was performed using the Kaplan–Meier survival curve and log-rank test. Any *P* value less than 0.05 was regarded as statistically significant.

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