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T cell augments the antitumor activity of tumor-targeting *Salmonella*

Abstract

Systemic administration of *Salmonella* to tumor-bearing mice leads to the preferential accumulation within tumor sites and retardation the tumor growth. However, the detailed mechanism of the *Salmonella*-induced antitumor immune response via host T cell remained uncertain. Herein, we used wild-type, $CD4^+$ T-cell-deficient, and $CD8^+$ T-cell-deficient mice to study the role of T cell in the antitumor immune responses induced by *Salmonella enterica* serovar Choleraesuis (*S.* Choleraesuis). When systemically administered into mice bearing tumors, *S.* Choleraesuis significantly inhibited tumor growth by 50%. By contrast, in T-cell-deficient mice, there was only 34%~42% in inhibition of tumor growth. We found that the treatment of *S.* Choleraesuis significantly upregulate interferon-γ in wild-type and $CD8^+$ T-cell-deficient mice, but not in $CD4^+$ T-cell-deficient mice. Furthermore, immunohistochemical staining of the tumors revealed more infiltration of macrophages, and neutrophils in wild-type mice after *S.* Choleraesuis treatment compared with those in T-cell-deficient mice. The antitumor therapeutic effect mediated by *S.* Choleraesuis is associated with an inflammatory immune response at tumor site and a tumor T helper 1-type immune response. In conclusion, these results suggest that tumor-targeted therapy using *S.* Choleraesuis, which exerts tumoricidal effects and stimulates T cell activities, represents a potential strategy for the treatment of tumor.

Keywords: T cell; *Salmonella enterica* serovar Choleraesuis; interferon-γ; tumor.

Introduction

The exploration of wild-type and genetically modified bacteria strains as potential therapeutics in the treatment of cancer has been inspired by the incidental observations of cancer regression following spontaneous bacterial infection. Studies in which cancer patients were infected with *Steptococcus pyogenes* were initiated by Busch in 1868, Fehleisen in 1883 and Coley in 1891(Pawelek et al. 2003) In 1964, a series of reports described the use of nonpathogenic *Clostridia* in experimental treatment of cancer in rodents (Engelbart and Gericke 1964; Thiele et al. 1964). The rationale for using *Clostridium* as an anticancer agent is based on the fact that the hypoxic regions of solid tumors provide anaerobic bacteria such as *Clostridium* a suitable environment to germinate and growth. The proliferation of the bacteria presumably occurs in the hypoxic and necrotic regions of the tumor, but not in well-oxygenated normal tissues. Although promising, this antitumor strategy may have major limitations. Tumor lysis is restricted to large, well-establish tumors but is undetectable in smaller metastatic deposits, probably because of the lack of hypoxic regions in these lesions. Apart from obligate anaerobes which target hypoxic/necrotic areas of solid tumors, *Salmonella typhimurium*, a facultative anaerobe capable of growing under both aerobic and anaerobic conditions, has also been exploited as a potential oncolytic agent (Pawelek et al.1997; Low et al. 1999). *Salmonellae* are gram-negative, facultative anaerobes that are a common cause of intestinal infections. As *Salmonellae* survive and replicate in both oxygenated and hypoxic conditions, they would be expected to colonize small metastatic lesions as well as larger tumors. Meanwhile, attenuated strains of *Salmonella* have a number of

desirable properties as tumor-targeted anticancer agents, which include targeting of tumors from a distant inoculation site, selective replication within tumors, and tumor growth suppression. Although *Salmonella* possesses properties of targeting of multiple tumors, selective replication within tumors, tumor retardation, and gene delivery, the mechanisms contributing to its initial infection to tumors, preferential accumulation within tumors to high levels relative to normal tissues, and oncolytic effects are largely not understood. There are some factors influenced the tumor colonization of *Salmonella*. It has been shown that *Salmonella* has limited ability to adhere to tumor vasculature and migrate within tumors and only survives in tissue that becomes necrotic (Forbes et al. 2003). The immune response against *Salmonella* is composed of an immediate response mediated by the innate arm of the immune system followed by antigen-specific adaptive immunity. Together, these two arms of immune system work to eradicate infection and provide long-lasting immunity and memory. To improve the efficacy and safety of the oncolytic therapy of *Salmonella*, a further understanding of *Salmonella* interactions with the immune system is required.

The attenuated *Salmonella enterica* serovar Choleraesuis (*S.* Choleraesuis) described here has been used previously as a live vaccine for swine and as a DNA vaccine delivery vector for mice (Shiau et al. 2001). However, its genetic characteristics have not been well defined. We previously reported to exploit an attenuated *S.* Choleraesuis as an antitumor agent capable of preferentially accumulating and amplifying within tumors, and as a gene delivery vector to transfer gene at the tumor site (Lee et al. 2004; Lee et al. 2005; Lee et al. 2008a). Auxotrophic *S.* Typhimurium has

been used as an antitumor agent in previous studies (Pawelek et al. 1997; Low et al. 1999). Yu et al. demonstrated that not only did attenuated bacterial strains, such as *S. typhimurium*, *Vibrio cholerae*, and *Listeria monocytogenes*, gain entry and replicate in the tumor tissue, *E. coli* DH5α strain also exhibited tumor-specific localization, indicating that no mutations affecting the survival of the bacteria are required for tumor-targeted potential (Yu et al. 2004).

The use of T-cell-deficient mice allowed us to analyze the role of T cells in tumor-bear mice after *Salmonella* administration. These results not only indicate that mechanisms that T cells are important for control of systemic *Salmonella* treatment, but also that T cells participate in antitumor activities of *Salmonella*. Herein, we want to identify that T cell involved in the antitumor effector of oncolytic bacteria by using *S.* Choleraesuis.

Material and methods

Cells, Bacteria and Mice

Lewis lung carcinoma (LL2) was cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 50 μg/ml gentamicin, 2 mM L-glutamine, and 10% heat-inactivated fetal bovine serum (FBS) at 37℃ in 5% CO_2. A vaccine strain of *S.* Choleraesuis (ATCC 15480) was obtained from Bioresources Collection and Research Center (Hsinchu, Taiwan). 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 strain Dublin over the surface of a dried nutrient agar plate and placing a drop of a suspension of salmonella anti-O phage no. 1, and selecting for a phage-resistant colony after incubation at 37^{o} C for 24 h (Lee et al. 2009). Male C57BL/6 mice at the age of 6 to 8 weeks were obtained from the Laboratory Animal Center of the National Cheng Kung University. Male $CD8^{+}$, and $CD4^{+}$ T -cell-deficient mice were purchased from the Jackson Laboratory. 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.

Animal Studies

The mice were inoculated subcutaneously (s.c.) with 10^6 tumor cells. When the tumors had grown to 50 mm^3 to 100 mm^3, the mice were injected intravenously (i.v.) with 2×10^6 cfu of *S.* Choleraesuis. At various time points postinfection, 3-4 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. In a separate experiment, palpable tumors were measured every 3 days in two perpendicular axes with a tissue caliper and the tumor volume was calculated as: (length of tumor) × $(\text{width of tumor})^2 \times 0.45$.

Assessment of Cytokines and Immunohistochemical Staining

To determine the expression of IFN-γ after *S.* Choleraesuis injection, mice were inoculated with LL2 cells (10^6) at day 0. Then, the groups of mice were treated with *S.* Choleraesuis (2×10^6 cfu) by intravenous injection at day 10. To detect the protein and cytokine expressions, the organs were collected at day 11. Levels of IFN-γ in the tissue homogenates or sera were determined by ELISA (R＆D, Minneapolis, MN). The protein content in each sample was determined by bicinchoninic acid (BCA) protein assay (Pierce Biotechnology, Rockford, IL). To analyze cell infiltrates in the tumors, groups of 4 mice that had been inoculated s.c. with 10^6 LL2 cells at day 0 were injected i.v.

with either 2×10^6 cfu of S. Choleraesuis at day 7. Control mice received PBS. The tumors were excised and snap frozen at time point. Cryostat sections (5 μm) were also prepared, fixed, and incubated with rat anti-mouse Ly-6G (Gr-1) (RB6-8C5, BD Biosciences, San Diego, CA), rat anti-mouse Mac-3 (M3/84, BD Biosciences), rat anti-mouse CD4 (L3T4) (GK1.5, BD Biosciences), or rat anti-mouse CD8a (Ly-2) (53-6.7, BD Biosciences) antibody. After sequential incubation with appropriate peroxidase-labeled secondary antibody and aminoethyl carbazole (AEC) as substrate chromogen, 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 × 200 magnification in each section (Lee et al. 2008a).

Statistical Analysis

The unpaired, two-tailed Student's* t *test was used to determine differences between groups for the comparisons of tumor volume, tumor weight, and the numbers of macrophages, and neutrophils. Any* p *value less than 0.05 is regarded statistically significant.

Results

Increased infiltrating $CD4^+$and $CD8^+$ T cells in the tumors following the treatment of *Salmonella*

The persistence of high levels of *Salmonella* within tumors may induce inflammatory responses, leading to the recruitment of immune cells to the tumor site (Lee et al. 2008a; Lee et al. 2004; Lee et al. 2005). The clearance of *Salmonella* from infected tissues is controlled by the acquisition of adaptive immunity. The tumors from mice bearing the LL2 tumors treated with *S.* Choleraesuis were analyzed for cell infiltrates by immunohistochemical staining. Representative results for immunohistochemistry are shown in Fig. 1a. Notable increases of $CD8^+$ and $CD4^+$ T cells that infiltrated into the tumors were observed in the mice treated with *S.* Choleraesuis (Fig. 1b). These results indicate that *S.* Choleraesuis resulted in increasing infiltrating $CD8^+$ and $CD4^+$ T cells in the tumors. As 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 T cells in *S.* Choleraesuis-treated tumors. The T cell response against tumor cells presumably enhanced the antitumor efficacy of *S.* Choleraesuis.

Tissue distributions of *Salmonella* in mice

Previously, We investigated the impact of host immune responses on the tumor-targeting potential of *S.* Choleraesuis, BALB/c and NOD/SCID mice bearing tumors were injected with *S.*

Choleraesuis, and the amounts of accumulated *S.* Choleraesuis in the tumors, livers, and spleens were determined at various time points (Lee et al. 2004). The bacterial amount was much higher in the tumors than in the livers and spleens at all the time points examined in both strains of mice. Their amounts in the tumors reached a peak level at day 2 and maintained in similar high levels during the first 6 days. Notably, bacterial load in the tumors was reduced by approximately 4 orders of magnitude in BALB/c mice at day 12, whereas there was no equivalent decrease in the bacterial number in NOD/SCID mice. Similarly, there was a decline in the bacterial numbers in the livers and spleens at 6 days and further decreased to undetectable levels in BALB/c mice at day 12. In contrast, in NOD/SCID mice bacterial levels in the three tissues remained fairly constant and persisted for at least 12 days, with constant 10000:1 ratio of bacterial accumulation in the tumor to accumulation in the liver or spleen. Therefore, our data, combined with those reported previously (Forbes et al. 2003), suggest that the abilities of *Salmonella* in initial localization and subsequent replication within tumors are not dependent on the host's immune system; however, by around 1 week when adaptive immune responses are elicited, *Salmonella* may be cleared more rapidly from livers and spleens, where abundant immune cells are present, than from tumors by host immune surveillance, thereby persisting longer within the tumor. Herein, to investigate the impact of T cell on the tissue distributions of *Salmonella*, the wild-type ,$CD4^+$ T-cell-deficient, and $CD8^+$ T-cell-deficient mice bearing tumors were injected with *S.* Choleraesuis and the amounts of *S.* Choleraesuis in the tumors, livers, and spleens were determined at day 1 and day10. As shown in Fig. 2, the bacterial amount was much higher in the tumors than other healthy organs at all the time

points in all examined mice. Their amounts in the tumors maintained in high levels during the 10 days. Notably, bacterial load in the tumors was less in wild-type mice than that in $CD4^+$ T-cell-deficient mice (Fig. 2a). The amounts of S. Choleraesuis in tumors were not significantly different between wild-type and $CD8^+$ T-cell-deficient mice. Taken together, these results suggest that $CD4^+$- T-cell influenced the tumor-targeting potential of *S.* Choleraesuis.

Antitumor effects of *Salmonella* on T-cell-deficient and wild-type mice

We next compared the antitumor effects of systemic administration of *S.* Choleraesuis on wild-type, $CD4^+$ T-cell-deficient, and $CD8^+$ T-cell-deficient mice bearing syngeneic LL2 tumor (Fig. 3). Twenty-two days after tumor inoculation, wild- type mice receiving *S.* Choleraesuis had small tumor volume than the control mice receiving PBS (50.46%, $p < 0.01$) (Fig. 3a). By contrast, there was a slight difference in mean tumor volumes in $CD4^+$ T-cell-deficient mice ($P < 0.05$) (Fig. 3b). The mean tumor volume in $CD4^+$ T-cell-deficient mice treated with *S.* Choleraesuis was lowered by 34.75% compared with that treated with PBS, but that was lowered by 42.15% in $CD8^+$ T-cell-deficient mice (Fig. 3b and c). To further quantify tumor burden, the tumor weight was measured. The wild- type mice receiving *S.* Choleraesuis significantly inhibited the growth of tumor compared with the control mice receiving PBS (62.17%, $p < 0.01$). The $CD4^+$ T-cell-deficient mice treated with *S.* Choleraesuis had 26.47% less tumor weight compared with those treated with PBS, but that was lowered by 44.63% in $CD8^+$ T-cell-deficient mice (Fig. 3d).

Effects of *Salmonella* on interferon-γ induction *in vivo*

Previously, we found that IFN-γ play an important role in antitumor activity of *Salmonella* treatment (Lee et al. 2008b). To examine the role of T cell on cytokine induction by *Salmonella*, wild-type and $CD4^+$ T-cell-deficient, and $CD8^+$ T cell-deficient mice were given an intravenous injection of *S.* Choleraesuis. IFN-γ levels of sera, spleens, liver, and tumors were measured (Fig. 4). The level of IFN-γ was not significantly changed in the sera, spleens, livers, and tumors derived from mice treated with PBS. Compared with $CD4^+$ T-cell-deficient mice that received *Salmonella*, the levels of IFN-γ were significantly increased in the sera, spleens, livers, and tumors derived from wild-type or $CD8^+$ T-cell-deficient mice treated with *S.* Choleraesuis. However, IFN-γ production induced by the bacteria was not observed in $CD4^+$ T-cell-deficient mice. Collectively, these findings demonstrate that *S.* Choleraesuis elicited IFN-γ for the most part via $CD4^+$ T cell.

Increased infiltrating immune cells in the tumors following the treatment of *Salmonella* in mice

The tumors from mice bearing the tumors treated with *S.* Choleraesuis were analyzed for cell infiltrates by immunohistochemical staining. Representative results for immunohistochemistry are shown in Fig. 5a. Notable increases of neutrophils, macrophages that infiltrated into the tumors

were observed in the mice treated with *S. Choleraesuis* and, in particular, in wild-type mice. The numbers of infiltrating immune cells in the tumors derived from $CD8^+$ T-cell-deficient mice treated with *S. Choleraesuis* were significantly increased compared with those in the tumors derived from $CD4^+$ T-cell-deficient mice treated with *Salmonella*, whereas no such difference was found in control mice treated with PBS (Fig. 5). Taken together, these results indicate that *S. Choleraesuis* increased more infiltrating immune cells in the tumors derived from $CD8^+$ T-cell-deficient mice than these derived from $CD4^+$ T-cell-deficient mice.

Discussion

The successful induction of immunity against poorly immunogenic malignancies is a major challenge for cancer therapy. Previously, we have demonstrated that host immune responses cooperate with bacteria-mediated tumor destruction during *S.* Choleraesuis treatment (Lee et al. 2004; Lee et al. 2005). The interactions between oncolytic *S.* Choleraesuis therapy and immune mechanisms are likely to be complex. In the present study, we have identified one important mechanism involved in the recruitment of effector immune cells. Previously, it was detected strong IFN-γ production in the tumors derived from mice after *S.* Choleraesuis treatment (Lee et al. 2008b). Herein, we demonstrate that *S.* Choleraesuis induced IFN-γ production and polarized the T-cell response to a Th1-dominant state in wild-type mice, but not in $CD4^+$ T-cell-deficient mice. Thus, bacteria-activated $CD4^+$ T cell infiltrating tumor may be a relevant source of IFN-γ in the tumor microenvironment and may contribute, a least in part, to the host antitumor immunity induced by *S.* Choleraesuis. Meanwhile, the accumulation of *Salmonella* in tumor sites provoked a potent inflammatory response, which recruited large numbers of immune cells. IFN-dependent chemokines, such as IFN-inducible chemokines CXCL9 (MIG), and CXCL10 (IP-10) (Lee et al. 2008a; Lee et al. 2008b), induced by *S.* Choleraesuis are expected to recruit activated effector cells within the tumor. Actuality, we found a large number of infiltrating immune cells such as macrophages, neutrophils within tumor microenvironment. Antitumor effects of neutrophils, in particular, after being activated by substances derived from microorganisms have also been demonstrated in various tumors (Kasai et al. 1991; Fujii et al. 1987; Yang et al. 1992). Strong neutrophils activation can

cause tissue damage and this represents the basis for tumor destruction. In addition, macrophages activated by bacterial products such as lipopolysaccharide (LPS) and Th1 cytokine are capable of lysing tumor cells, expressing immunostimulatory cytokines, and presenting tumor-associated antigens to recruit T cells (Luo et al. 2006). Recently, the $CD8^+$ T cell immune response induced by *Salmonella* reported by Saccheri et al. was observed antimicrobial response present in tumors to activate cytotoxic $CD8^+$ T cells that could recognize and kill tumor cells (Saccheri et al. 2010). In our study, we also demonstrated that $CD8^+$ T cell is involved in the antitumor effector of *S.* Choleraesuis. The current findings and our previous reports strongly suggest that *Salmonella* elicit antitumor effects by stimulating host immune responses (Lee et al. 2004; Lee et al. 2008b; Saccheri et al. 2010).

In $CD8^+$ T-cell deficient mice, we also observed the antitumor effect of *S.* Choleraesuis, the slight production of IFN-γ and the infiltrating immune cells in the tumors. These results implicate that the antitumor effectors of *S.* Choleraesuis is partially mediated through $CD4^+$ T cell. Furthermore, LPS from *Salmonella* may induce apoptosis of tumor and endothelial cells to enhance the activity of antitumor (Bannerman et al. 2001). Meanwhile, the competition for nutrients between tumor cells and *Salmonella* may also induce the suppression of tumor growth (Pawelek et al. 1997). The mechanisms involved in the antitumor effects of *S.* Choleraesuis are likely to be multifaceted. Thus, further work is warranted to elucidate the more underlying mechanism of antitumor effects of *S.* Choleraesuis.

In the present study, we show that *Salmonella* significantly upregulated IFN-γ which may be

responsible for recruiting peripheral immune cells to the tumor in wild-type mice, but not in T-cell-deficient mice. We suggest the T cell is involved in the regulation of *Salmonella*-induced host antitumor immunity in tumor-bearing mice. Thus, this study may provide a cellular basis for understanding the recruitment of effector immune cells and the synergism between the oncolytic effect of *S.* Choleraesuis and adaptive antitumor immune mechanisms. It is possible to exploit an immune response to infection to inhibit tumor growth by bypassing tumor immune evasion mechanism (Saccheri et al. 2010). In phase I study, patients received VNP20009 that rapidly cleared from blood, and most tumor were not detectable the colonization of *Salmonella* (Toso et al. 2002). Patient had pre-existing anti-*Salmonella* antibodies and was not accompanied the colonization of *Salmonella* in the tumor sites after systemically administration. In agreement with clinical study, our previous results indicated that the higher anti-*Salmonella* antibody titers in the host cause fewer amounts of *Salmonella* in the tumor sites (Lee et al. 2009). In this work described here, we suggest that host T cells also play important roles in the tumor-reducing effect of *Salmonella* in tumor-bearing mice. Previously, we demonstrated that *S.* Choleraesuis in combination with cisplatin appears to hold promise for the treatment of solid tumors (Lee et al. 2005). The combination therapy may facilitate the bacterial therapies with improve antitumor efficacy. Herein, this new understanding of the mechanisms of antitumor activities of *Salmonella* in host will allow for the development of safe and efficient bacterial therapies in future clinical trials.

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FIGURE LEGEND

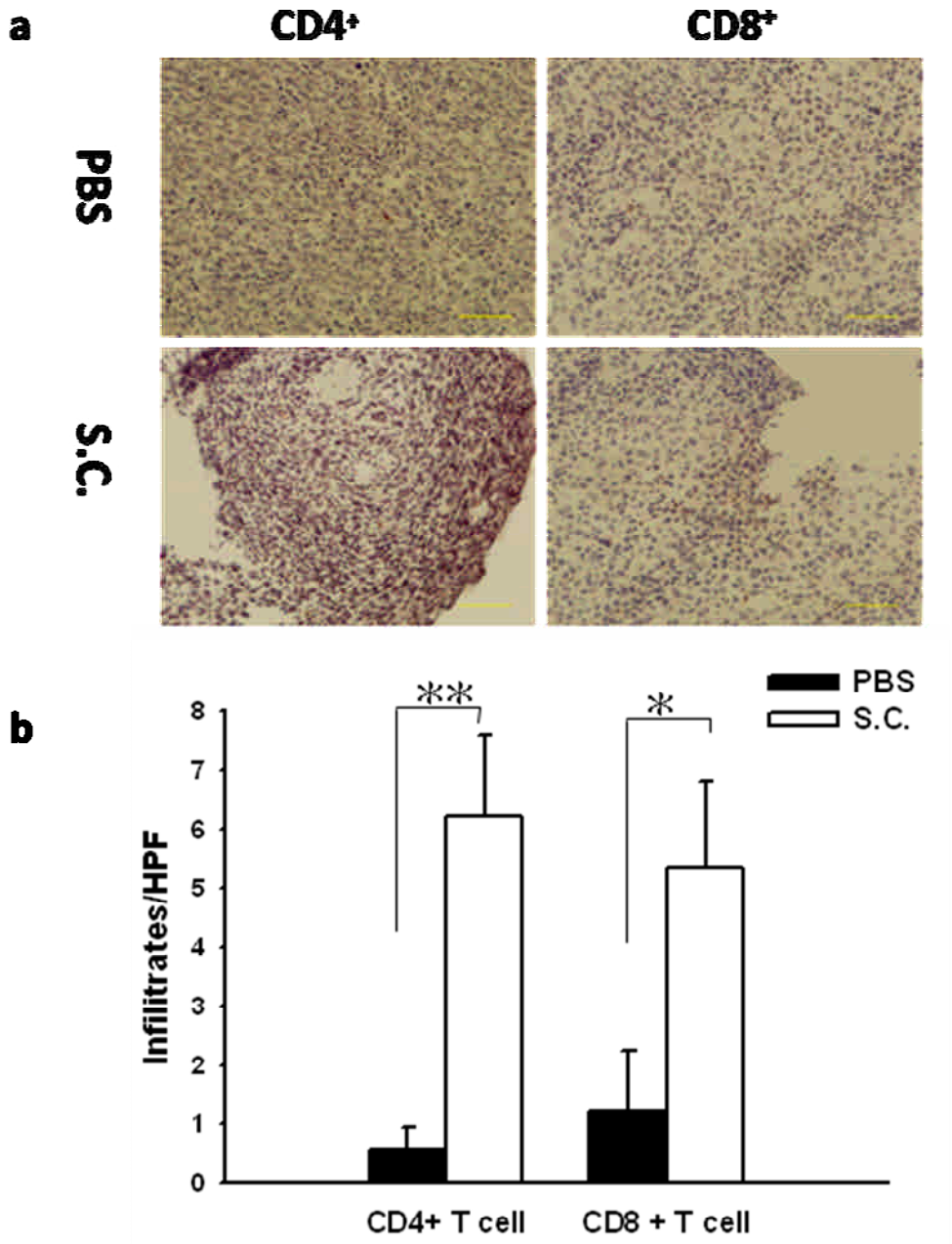

Fig. 1. Increases in $CD4^+$ T- and $CD8^+$ T-cell infiltrates in the tumors from LL2 tumor-bearing mice treated with *S.* Choleraesuis (S.C.). Groups of 4 C57BL/6 mice that had been inoculated s.c. with LL2 cells (10^6) at day 0 were treated i.v. with *S.* Choleraesuis (2×10^6 cfu) at day 7. Vehicle control mice received PBS. A, Tumors were excised at day 20 and immunostained with antibodies against $CD4^+$ or $CD8^+$ (× 200). B, $CD4^+$ and $CD8^+$ T cells that infiltrated tumors were determined by

averaging the cell numbers from three fields of highest positive-stained cell density at × 200 magnification in each section (mean ± SEM, n=4).*, $P < 0.05$; **, $P < 0.01$. Each experiment was repeated three times with similar results.

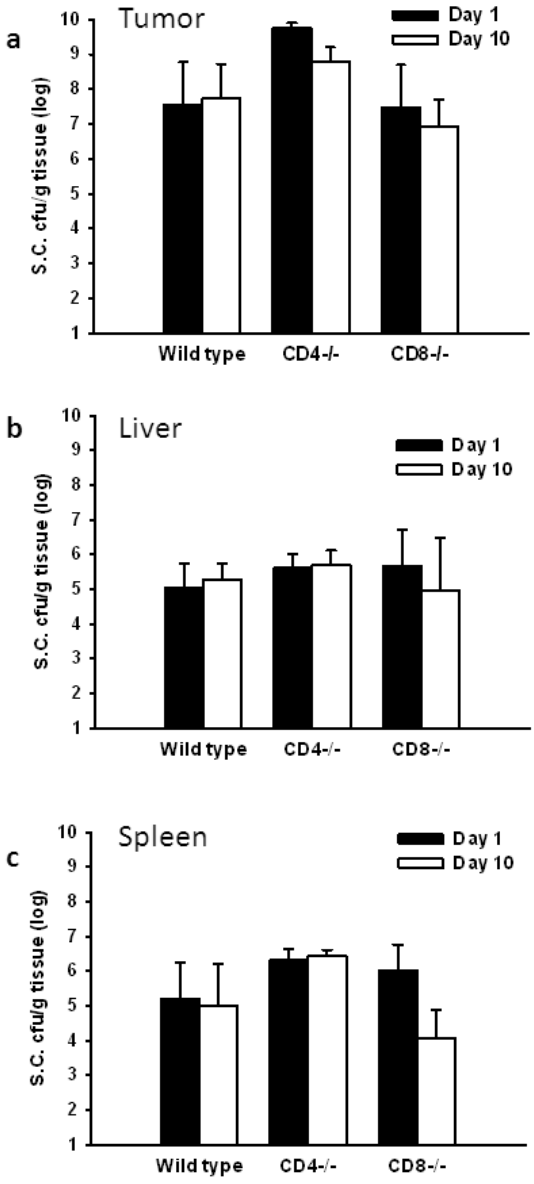

Fig. 2. The spatial and temporal distribution of *S.* Choleraesuis (S.C.) in T-cell-deficient and wild-type mice. The mice bearing LL2 tumors ranging from 50 to100 mm^3 were injected i.v. with *S.* Choleraesuis (2×10^6 cfu), and the amounts of *S.* Choleraesuis in the (A) tumor, (B) livers, and (C) spleens were determined at 1 and 10 day (mean ± SD, n = 3-4) postinfection. Each experiment was

repeated three times with similar results.

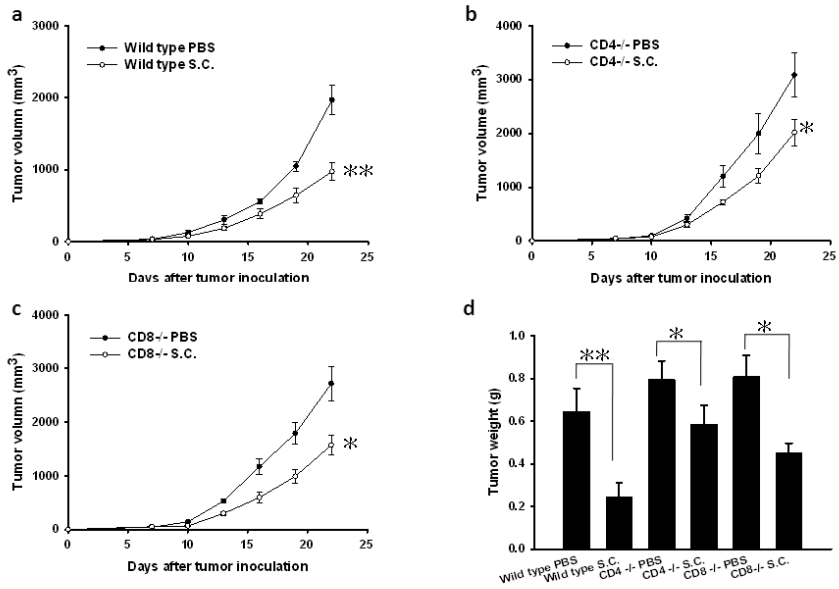

Fig. 3. Antitumor effects of *S.* Choleraesuis (S.C.) on tumor growth in T-cell-deficient and wild-type mice bearing LL2 tumors. A, Groups of 7 mice that had been inoculated s.c. with LL2 cells (10^6) at day 0 were treated i.v. with *S.* Choleraesuis (2×10^6 cfu) or PBS at day 7. B, Groups of 7 $CD4^+$ T-cell-deficient mice that had been inoculated s.c. with LL2 cells (10^6) at day 0 were treated i.v. with S. Choleraesuis (2×10^6 cfu) or PBS at day 7. (C) Groups of 6-7 $CD8^+$ T-cell-deficient mice that had been inoculated s.c. with LL2 cells (10^6) at day 0 were treated i.v. with S. Choleraesuis (2×10^6 cfu) or PBS at day 7.Tumor volumes among different treatment groups were compared at day 22. (D) Groups of 3-4 mice that had been inoculated s.c. with LL2 cells (10^6) at day 0 were treated i.v. with *S.* Choleraesuis (2×10^6 cfu) or PBS at day 7. The tumor weight was measured at day 10 postinfection.

*, $P < 0.05$; **, $P < 0.01$. Each experiment was repeated three times with similar results.

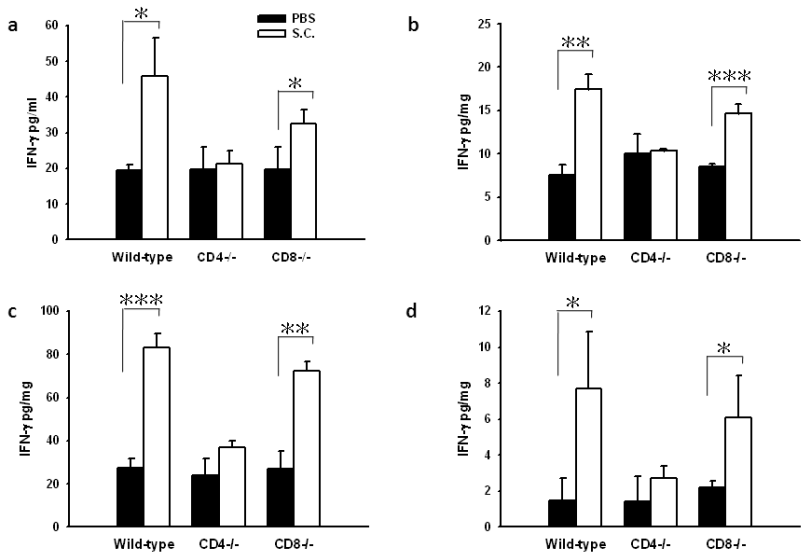

Fig. 4. Effects of *S.* Choleraesuis (S.C.) on cytokine induction in T-cell-deficient and wild-type mice bearing LL2 tumors. The mice bearing LL2 tumors injected i.v. with *S.* Choleraesuis (2×10^6 cfu). IFN-γ levels in the (A) sera, (B) liver, (C) spleens, and (D) tumors were measured by ELISA at 12 h postinfection (mean ± SD, n = 3-4). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. Each experiment was repeated three times with similar results.

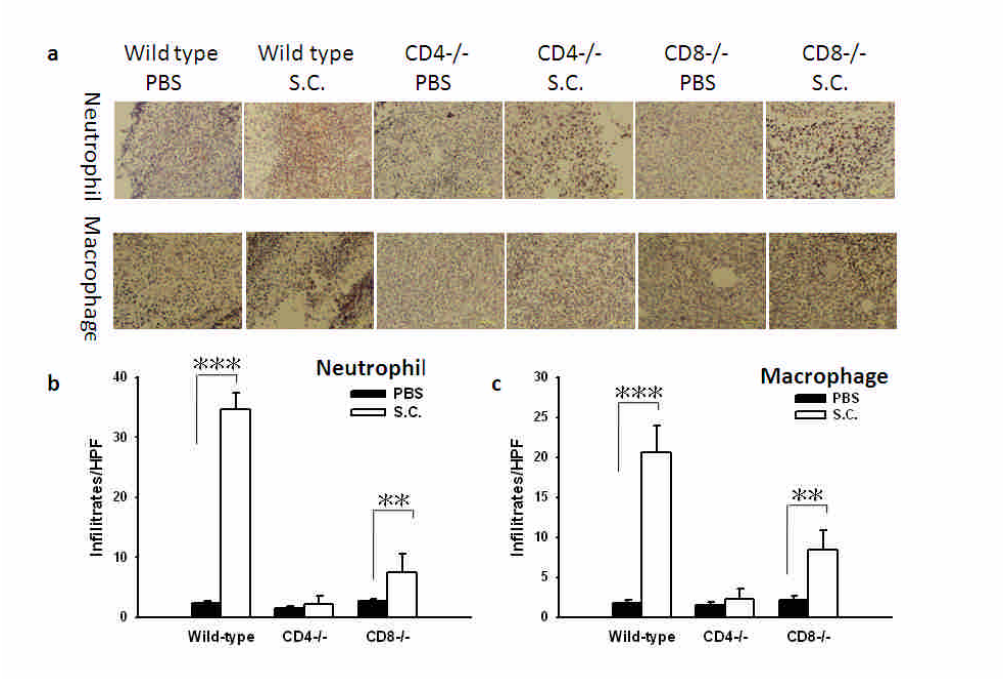

Fig. 5. Increase of infiltrating cells in the tumors from T-cell-deficient mice treated with *S.* Choleraesuis (S.C.). Mice bearing LL2 tumors at day 0 were injected i.v. with 2×10^6 cfu of *S.* Choleraesuis or with PBS at day 7. A, Tumors were excised at day 20, immunostained with antibodies against Gr-1, and Mac-3 were used to detect infiltrating cells (× 200). (B) Neutrophils, and (C) macrophages cells that infiltrated tumors were determined by averaging the cell numbers from three fields of highest positive-stained cell density at × 200 magnification in each section (mean ± SEM, n =4). **, $P < 0.01$; ***, $P < 0.001$. Each experiment was repeated three times with similar results.