Toll-like Receptor 4 Mediates an Antitumor Host Response Induced by *Salmonella choleraesuis*

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Abstract

**Purpose:** We have shown tumor-targeting and antitumor activities of an attenuated *Salmonella choleraesuis* in various tumor models. Meanwhile, host factors, including innate and adaptive immune responses, play roles in *Salmonella*-induced antitumor activity. Toll-like receptor 4 (TLR4) is identified as a signaling receptor for lipopolysaccharide derived from Gram-negative bacteria. However, the detailed mechanism of the *S. choleraesuis*–induced antitumor immune response via TLR4 remains uncertain.

**Experimental Design:** Herein, we used wild-type C3H/HeN mice and TLR4-deficient C3H/HeJ mice to study the role of TLR4 in the antitumor immune responses induced by *S. choleraesuis*.

**Results:** The amounts of *S. choleraesuis* were cleared more rapidly from the normal organs in C3H/HeN mice than those in C3H/HeJ mice. Tumors in C3H/HeN mice treated with *S. choleraesuis* were significantly smaller than those treated with PBS. By contrast, in TLR4-deficient mice, there was a slight difference in inhibition of tumor growth. Meanwhile, we found that *S. choleraesuis* significantly up-regulated IFN-γ, IFN-inducible chemokines CXCL9 (MIG), and CXCL10 (IP-10) productions in C3H/HeN mice, but not in C3H/HeJ mice. Furthermore, immunohistochemical staining of the tumors revealed less intratumoral microvessel density, more infiltration of macrophages, neutrophils, CD4+ and CD8+ T cells, and cell death in C3H/HeN mice after *S. choleraesuis* treatment compared with those in C3H/HeJ mice. The interaction between TLR4 and *S. cholerae- suis* seemed to polarize the T-cell response to a T helper 1–dominant state.

**Conclusions:** These results suggest TLR4 may play an important role in the molecular mechanism of *S. choleraesuis*–induced host antitumor responses.

Beneficial effects of bacterial infection on tumors have been observed since the 18th century (1). *Salmonella typhimurium*, a facultative anaerobe capable of growing under both aerobic and anaerobic conditions, has also been exploited as a potential oncolytic agent (1, 2). Previously, we exploited an attenuated *Salmonella choleraesuis* as a tumoricidal agent and a vector to deliver antiangiogenic genes for tumor-targeted gene therapy. We showed its tumor-targeting potential in various syngeneic murine tumor models (3–5). Recently, we exploited *S. choleraesuis* as a single tumor-targeting anticancer agent and as part of a combination therapy with low-dose cisplatin for mice bearing syngeneic tumors, including metastatic and orthotopic tumors (5). The tumor-targeting potential of *S. choleraesuis* probably elicits antitumor effects by stimulating immunocompetent cells, such as T cells and neutrophils (5, 6). However, the detailed antitumor immune response mechanism induced by *S. choleraesuis* remains uncertain.

Toll-like receptors (TLR) are mammalian homologues of *Drosophila* toll receptor that play an essential role in the innate recognition of microorganisms by the host. Toll-like receptor 4 (TLR4) is recently identified as a signaling receptor for lipopolysaccharide derived from Gram-negative bacteria, such as *Salmonella*. After lipopolysaccharide binding, TLR4 initiates signals through the sequential recruitment of myeloid differentiation protein 88 and tumor necrosis factor− receptors–associated factor 6, which in turn activate downstream mediators, such as mitogen-activate protein kinase and nuclear factor-κB, and lead to the activation of genes encoding proinflammatory cytokines (7, 8). It is of interest to explore the hypothesis that the function of TLR4 contributes to the antitumor immune responses of the host induced by *S. choleraesuis*.

In this study, we investigate the roles of innate and adaptive immune responses in the antitumor immune responses elicited by *Salmonella* infection and especially focus on the contribution of TLR4 in *Salmonella*-mediated antitumor activities. However, we examined the effect of TLR4 on the spatial and temporal distribution of *S. choleraesuis* and compared the antitumor effects of *S. choleraesuis* in C3H/HeN (wild type) and C3H/HeJ (TLR4 deficiency) mice. *S. choleraesuis* induces IFN-γ and polarizes the T-cell response to a Th helper 1 (Th1)–dominant state in C3H/HeN mice.
We showed that the accumulation of tumor by S. choleraesuis was associated with up-regulation of IFN-inducible chemokines as potent chemoattractants for lymphocytes and antiangiogenic agent (6, 9, 10). S. choleraesuis elicited Th1 cytokine-inducing, chemokine expression, and antitumor activities via TLR4 signaling, a finding which may help clarify the molecular mechanism of S. choleraesuis-induced antitumor host responses.

**Materials and Methods**

Cell lines, bacteria, and mice. Murine K1735 melanoma cells were cultured in DMEM supplemented with 50 μg/mL gentamicin, 2 mmol/L L-glutamine, and 10% heat-inactivated fetal bovine serum at 37°C in 5% CO₂. A vaccine strain of S. choleraesuis [S. choleraesuis subsp. choleraesuis (Smith) Weldin serovar Dublin (ATCC 15480)] was obtained from Bioresources Collection and Research Center (11). Male C3H/HeN and C3H/HeJ mice at ages 6 to 8 wk 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.

Animal studies. C3H/HeN and C3H/HeJ mice were inoculated s.c. with 10⁶ K1735 cells. When the tumors had grown to 50 to 100 mm³, which took around 7 d to reach, the mice were injected i.p. with 2 × 10⁶ colony-forming units (cfu) of S. choleraesuis. At various time points postinfection, three to four 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 (5). In a separate experiment, palpable tumors were measured every 4 d in two perpendicular axes with a tissue caliper, and the tumor volume was calculated as (length of tumor) × (width of tumor)² × 0.45.

Assessment of cytokines and chemokines. The levels of IFN-γ and interleukin-4 in the sera, spleens, and tumors after S. choleraesuis administration were determined by ELISA (R&D Systems). The levels of MIG and IP-10 mRNA in K1735 tumors after S. choleraesuis...
administration were determined by reverse transcription–PCR. Total cellular RNA was isolated and reverse transcribed into cDNA using standard methods. The specific primer pairs used for detecting mouse IFN-inducible chemokines CXCL9 (MIG), CXCL10 (IP-10), and h-actin were 5'-ACT CAG CTC TGC CAT GAA GTC CG and 5'-AAA GGC TGC TCT GCC AGG GAA GG, 5'-ATG AAC CCA AGT GCT GCC GTC and 5'-TTA AGG AGC CCT TTT AGA CCT TT, and 5'-TGG AAT CCT GTG GCA TCC ATG AAA C and 5'-TAA AAC GCA GCT CAG TAA CAG TCC G, respectively (6).

Immunofluorescence and immunohistochemical staining and apoptotic assays. To analyze cell infiltrates in the tumors, groups of four C3H/HeN or C3H/HeJ mice that had been inoculated s.c. with 10⁶ K1735 cells at day 0 were injected i.p. with either 2×10⁶ cfu of S. choleraesuis at day 7. Control mice received PBS. The tumors were excised and snap frozen at day 14. For immunofluorescence staining of IFN-γ and CD3⁺ cells, tumor sections were fixed in 10% formalin, permeabilized with cold acetone, incubated with rat anti-mouse CD3e⁺ antibody (145-2C11, PharMingen) and goat anti-mouse IFN-γ antibody (D-17, Santa Cruz Biotechnology) at room temperature for 40 min, and subsequently incubated with fluorescein-conjugated goat anti-rat IgG (KPL) and rhodamine-conjugated rabbit anti-goat IgG (Jackson). Nuclei were stained with 50 μg/mL of 4',6-diamidino-2-phenylindole. Expression and localization of the proteins were observed under fluorescence microscope at magnification of 400×. Cryostat sections (5 μm) were also prepared, fixed, and incubated with rat anti-mouse Ly-6G (Gr-1; RB6-8C5, PharMingen), rat anti-mouse Mac-3 (M3/84, PharMingen), 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 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 400× magnification in each section. Tumor vascularization was determined by counting factor VIII–positive vessels (DAKO) with immunohistochemical analysis. Microvessel density was determined by averaging the number of microvessels in three areas of highest vessel density at 400× magnification in each section (3, 4). The terminal deoxynucleotidyltransferase-mediated dUTP nick end-labeling (TUNEL) assay was used to detect cell apoptosis within tumors and was done according to the manufacturer’s instructions (Promega). TUNEL-positive cells were determined under the microscope and counted as previously described (5, 6).

Statistical analysis. The unpaired, two-tailed Student’s t test was used to determine differences between groups for the comparisons of tumor volume, the level of cytokines, and the numbers of S. choleraesuis, neutrophils, macrophages, CD4⁺, CD8⁺ T cells, factor VIII–positive cells, and apoptotic cells. Any P value of <0.05 is regarded statistically significant.

Results

Tissue distributions of S. choleraesuis in wild-type and TLR4-deficient mice. To investigate the effect of TLR4 on the tissue distributions of S. choleraesuis, C3H/HeN and C3H/HeJ mice bearing tumors were injected with S. choleraesuis, and the amounts of S. choleraesuis in the blood, tumors, livers,
spleens were determined at various time points. As shown in Fig. 1, 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 maintained in high levels during the 30 days. Notably, bacterial load in the tumors was slightly reduced in C3H/HeN mice at day 20, whereas there was no equivalent decrease in the bacterial number in C3H/HeJ mice. Even at day 10, *S. choleraesuis* could still be detected in the blood from C3H/HeJ mice. In contrast, *S. choleraesuis* was undetectable in the blood from C3H/HeN mice at day 10. Taken together, these results suggest that TLR4 signaling influenced the tissue distributions of *S. choleraesuis*.

**Antitumor effects of *S. choleraesuis* on TLR4-deficient and wild-type mice.** We next compared the antitumor effects of systemic administration of *S. choleraesuis* on C3H/HeN and C3H/HeJ mice bearing syngeneic K1735 melanoma (Fig. 2). Twenty-seven days after tumor inoculation, C3H/HeN mice receiving *S. choleraesuis* had small tumor volume than the control mice receiving PBS (*P* < 0.001). By contrast, there was a slight difference in mean tumor volumes in C3H/HeJ mice (*P* < 0.05). The mean tumor volume in C3H/HeN mice treated with *S. choleraesuis* was lowered by 57.79% compared with that treated with PBS, but that was lowered by 34.19% in C3H/HeJ mice.

**Effects of *S. choleraesuis* on cytokines and chemokines induction in vivo.** To examine the role of TLR4 on cytokine induction by *S. choleraesuis*, C3H/HeN and C3H/HeJ mice were given an i.p. injection of *S. choleraesuis*. After 12 hours, sera, spleens, and tumors IFN-γ levels were measured (Fig. 3A). The level of IFN-γ was not significantly changed in the sera, spleens, and tumors derived from C3H/HeN and C3H/HeJ mice treated with PBS. Compared with C3H/HeN mice that received PBS, the levels of IFN-γ were significantly increased in the sera, spleens, and tumors derived from C3H/HeN mice treated with *S. choleraesuis*. However, IFN-γ production induced by the bacteria was not significantly observed in C3H/HeJ mice. The levels of interleukin-4 in the sera, spleens, and tumors were not influenced by *S. choleraesuis* administration in both strains of mice (Fig. 3A). Because T cells are major IFN-γ–secreting cells, we next examined whether the secretion of IFN-γ was derived from the infiltrating T cells in the tumor microenvironment by immunofluorescence double staining with anti–IFN-γ antibody by rhodamine and anti-CD3+ antibody by fluorescein (Fig. 3B). Regardless of mice strains, T cells and IFN-γ were not significantly detected in tumors derived from mice treated with PBS. A small amount of T cells was detected in the tumors derived from C3H/HeJ mice treated with *S. choleraesuis*, whereas it was detected with strong signals in the tumors derived from C3H/HeN mice after *S. choleraesuis* treatment. As expected, IFN-γ was found mainly in T cells and their peripheral. Constant with the number of infiltrating T cells,
IFN-γ also have stronger staining in the tumors derived from C3H/HeN mice than C3H/HeJ mice. To further investigate the importance of IFN-γ in S. choleraeuis–mediated antitumor response, induction of chemokines by IFN-γ in the tumors sites were determined by reverse transcription–PCR in the meanwhile. We found significant inductions of MIG and IP-10 mRNA expressions in the tumors derived from C3H/HeN mice, but not in the tumors derived from C3H/HeJ mice, treated with S. choleraeuis (Fig. 4A). However, MIG and IP-10 are potent inhibitor of angiogenesis. The intratumoral microvessels in S. choleraeuis–treated mice bearing tumors were examined by immunohistochemistry. The results of immunohistochemical staining are given in Fig. 4B. Tumors derived from S. choleraeuis–treated C3H/HeN mice seemed much less vascularized than PBS-treated counterparts. As shown in Fig. 4C, the number of factor VIII–positive intratumoral microvessels in the C3H/HeN mouse treated with S. choleraeuis was significantly reduced 70.26% compared with that in C3H/HeN mice treated with PBS. On the contrary, the microvessels in the tumors derived from C3H/HeJ mice treated with S. choleraeuis were only reduced 35.22% in comparison with those derived from C3H/HeJ mice treated with PBS. Collectively, these findings show that S. choleraeuis elicited IFN-γ and IFN-γ–inducing responses via TLR 4 signaling.

**Increased infiltrating immune cells and apoptotic cells in the tumors after treatment of S. choleraeuis in mice.** The tumors from mice bearing the tumors treated with S. choleraeuis were analyzed for cell infiltrates by immunohistochemical staining and for apoptotic cells by TUNEL assay. Representative results for immunohistochemistry are shown in Figs. 5A and 6A. Notable increases of neutrophils, macrophages, CD4+ T cells, and CD8+ T cells that infiltrated into the tumors were observed in the mice treated with S. choleraeuis and, in particular, in C3H/HeN mice. The numbers of infiltrating immune cells in the tumors derived from C3H/HeN mice treated with S. choleraeuis were significantly increased compared with those in the tumors derived from C3H/HeJ mice treated with S. choleraeuis, whereas no such difference was found between C3H/HeN and C3H/HeJ mice treated with PBS (Fig. 5B-E). TUNEL assay shows an increase in the amount of cells undergoing apoptosis in the S. choleraeuis–treated mice compared with those derived from C3H/HeN mice treated with S. choleraeuis (Fig. 6A and B). Notably, S. choleraeuis could also promote tumor apoptosis in C3H/HeJ mice. Nevertheless, there was a 1.6-fold increase in the number of apoptotic cells induced by S. choleraeuis in C3H/HeN mice compared with that induced by S. choleraeuis in C3H/HeJ mice (Fig. 6B). Taken together, these results indicate that S. choleraeuis increased more infiltrating immune cells and cell death in the tumors derived from C3H/HeN mice than these derived from C3H/HeJ mice.

**Discussion**

The successful induction of immunity against poorly immunogenic malignancies is a major challenge for cancer therapy. Previously, we have shown that host immune responses cooperate with bacteria-mediated tumor destruction during S. choleraeuis treatment (3, 5, 6). The interactions between oncolytic S. choleraeuis 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. We show that S. choleraeuis induced IFN-γ production and polarized the T-cell response to a Th1-dominant state in TLR4 wild-type mice, but not in TLR4-deficient mice. The accumulation of S. choleraeuis in tumor sites provoked a potent inflammatory response, which recruited large numbers of immune cells. IFN-dependent chemokines, such as MIG and IP-10, induced by S. choleraeuis are expected to recruit activated effector cells within the tumor. Actually, we...
found a large number of infiltrating immune cells, such as macrophages, neutrophils, and T cells within tumor microenvironment. Antitumor effects of neutrophils, in particular, after being activated by substances derived from microorganisms have also been shown in various tumors (12–14). In addition, macrophages activated by bacterial products, such as lipopolysaccharide and Th1 cytokine are capable of lysing tumor cells, expressing immunostimulatory cytokines, and presenting tumor-associated antigens to recruit T cells (15). In our study, we showed that TLR4 signaling is involved in the antitumor effect of *S. choleraesuis*. The current findings and our previous reports strongly suggest that *S. choleraesuis* elicit antitumor effects by stimulating host immune responses (3–6).

However, the MIG and IP-10 are the CXC chemokines that lack the sequence Glu-Leu-Arg (ELR motif) at their NH₂ termini.
terminus. Unlike ELR-positive CXC chemokines, MIG and IP-10 are potent inhibitors of angiogenesis, tumor growth, and metastasis (9, 16). It exerts angiostatic activity by inhibiting endothelial cell migration, proliferation, or differentiation induced by vascular endothelial growth factor and fibroblast growth factors (9, 10, 16). Jia et al. found that the treatment of S. typhimurium VNP20009 significantly reduced both intratumoral microvessel density and tumoral vascular endothelial growth factor level and suggested that VNP20009 may inhibit tumor angiogenesis and growth (17, 18). Herein, we may provide the molecular mechanism of antiangiogenesis effects via host TLR4 signaling after Salmonella treatment.

In C3H/HeJ 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 TLR4. Recent studies show that flagellin isolated from Salmonella is recognized by TLR5 and has the antitumor activity (19). The TLR9 ligand, microbial products, such as CpG-DNA, promotes Th1 polarization and exerts an antitumor effect in experimental models (20). Interestingly, our TUNEL assay revealed that S. choleraesuis treatment increases the number of apoptosis cells in the tumors of C3H/HeJ mice (Fig. 6). Lipopolysaccharide from Salmonella may induce apoptosis of tumor and endothelial cells to enhance the activity of antitumor (21). Meanwhile, the competition for nutrients between tumor cells and S. choleraesuis may also induce the suppression of tumor growth (2). 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 S. choleraesuis significantly up-regulated IFN-γ, IFN-inducible chemokines MIG, and IP-10, which may be responsible for recruiting peripheral immune cells to the tumor and antiangiogenesis effects in C3H/HeN mice, but not in C3H/HeJ mice. We suggest the TLR4 is involved in the regulation of S. choleraesuis–induced host antitumor immunity in tumor-bearing mice. Thus, this study may provide a molecular basis for understanding the recruitment of effector immune cells and the synergism between the oncolytic effect of S. choleraesuis and innate and adaptive antitumor immune mechanisms.

References
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