B cells are required for tumor-targeting *Salmonella* in host

Che-Hsin Lee,[1*] Jeng-Long Hsieh,[2] Chao-Liang Wu,[3] Hui-Chun Hsu,[4] and Ai-Li Shiau[5*]

[1]Department of Microbiology, School of Medicine, China Medical University, Taichung, Taiwan

[2]Department of Nursing, Chung Hwa University of Medical Technology, Tainan, Taiwan

[3]Department of Biochemistry and Molecular Biology, National Cheng Kung University Medical College, Tainan, Taiwan

[4]Center for General Education, Chang Gung Institute of Technology, Tao-Yuan , Taiwan

[5]Department of Microbiology and Immunology, National Cheng Kung University Medical College, Tainan, Taiwan

Address correspondence and reprint requests to: Dr. Che-Hsin Lee, Department of Microbiology, School of Medicine, China Medical University, 91 Hsueh-Shih Road, Taichung 40402, Taiwan. E-mail: chlee@mail.cmu.edu.tw; Fax: +886-422053764; Tel:+886-4-22053366 ext:2173

[*] These two authors contributed equally to this work.

Abstract

Systemic administration of *Salmonella* to tumor-bearing mice leads to the preferential accumulation within tumor sites and retardation the tumor growth. Host factors including innate and adaptive immune responses influence *Salmonella*-induced antitumor activity. Antitumor activities of *Salmonella* are not only determined by the tumor regression but also by the host immune response. Herein, we demonstrated that B-cell plays an important role in the antitumor activity mediated by *Salmonella*. Body weight and survival of B-cell-deficient mice were decreased compare with wild-type, $CD8^+$-cell-deficient or $CD4^+$-cell-deficient mice after *Salmonella* administration. Although *Salmonella* accumulated within tumors in B-cell-deficient mice, the bacterial loads of healthy organs were higher than those in wild-type mice. The inflammation cytokine and bacteremia were found in B-cell-deficiency mice after *Salmonella* treatment. When *Salmonell*a accumulated within the tumor, B-cells inhibited the dissemination of *Salmonella* to other healthy organs. The depletion of host B-cells resulted in a noticeably higher total number of *Salmonella* in tumor and inhibited tumor growth. Meanwhile, B-cell depletive and adoptive transfer of serum experiments demonstrated that natural antibody produced by B-cell takes part in the control of *Salmonella* dissemination in tumor-bearing mice. In this study, we want to address the mechanisms of incorporating host immunoresponse as a way to augment

the antitumor activities of *Salmonella*.

Keywords: B cell; *Salmonella*; tumor-targeting; natural antibody.

Introduction

The exploration of wild-type and genetically modified bacterial strains as potential therapeutics in the treatment of cancer has been inspired by the incidental observations of cancer regression following bacterial infection (Pawelek et al. 2003). The use of preferentially replicating bacteria as an oncolytic agent is an innovative approach for treatment of tumor. 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). 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, *Salmonella* has a number of desirable properties as a tumor-targeted anticancer agents, which include targeting of tumors from a distant inoculation site, selective replication within tumors, tumor growth suppression, and ability to express therapeutic genes such as herpes simplex thymidine kinase (Pawelek et al.1997), *E. coli* cytosine deaminase (King et al. 2002) and cytokines (Yuhua et al. 2001). 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 in tumors, preferential accumulation within tumors

to high levels relative to normal tissues, and oncolytic effects are largely not understood.

It has been previously shown that toll-like receptor (TLR) 4 is involved in the regulation of *Salmonella*-induced host antitumor immunity in tumor-bearing mice (Lee et al. 2008b). Recent studies demonstrate that flagellin isolated from *Salmonella* is recognized by TLR5 and has the antitumor activity (Calcaterra et al. 2006). The distribution of *Salmonella* is restricted within the necrotic areas of tumor by neutrophils (Westphal et al. 2008). These indicate that the host immune response in tumors after colonization by *Salmonella* is highly complex. However, following *Salmonella* administration, a strong immune response may develop in the acute stage of infection. Previously, we demonstrated that specific anti-*Salmonella* antibodies significantly retard the antitumor activities of oncolytic *Salmonella* (Lee et al. 2009). Herein, we want to further explore whether the B-cells play an important role in the control of distribution of *Salmonella* in tumor-bearing mice after systemic *Salmonella* administration.

Material and methods

Cell lines, bacteria and mice

Murine Lewis lung carcinoma (LL2) were 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. The attenuated *S.* Choleraesuis (ATCC 15480) was obtained from Bioresources Collection and Research Center (Hsinchu, Taiwan) (Smith 1965). 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 $CD8^+$-deficiency, $CD4^+$ -deficiency and igh-6^{tm1Cgn} (B-cell deficiency) 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 wild-type and gene deficiency mice were injected intravenously with 2×10^6 cfu of *Salmonella*. The body weight and survival of mice were monitored daily. The mice were inoculated subcutaneously with 10^6 tumor cells. When the tumors had grown to 50 mm^3 to 100 mm^3, the mice were injected intravenously or intratumorally with 2 ×

10^6 cfu of *Salmonella*. At various time points postinfection, mice in each group were sacrificed, and the numbers of *Salmonella* in the tumors, livers, and spleens were determined on LB plates and expressed as cfu per gram of tissues. For *in vivo* B-cell-depleted experiments, mice that had been inoculated with LL2 cells at day 0 were injected intraperitoneally with anti-mouse B220 (RA3-6B2) monoclonal antibodies at day 8. The specific depletion was more than 98% as determined by flow cytometry. The mice in each group were sacrificed, and the organs were harvested at day 9. The numbers of *Salmonella* in the tumors, livers, spleens, and blood were determined. A parallel experiment was performed using B-cell-depleted mice inoculated with *Salmonella* at day 8, and palpable tumors were measured every 4 days in two perpendicular axes with a tissue caliper and the tumor volume was calculated as: (length of tumor) $\times$ (width of tumor)^2 $\times$ 0.45. To investigate the effects of natural antibody on *Salmonella* infection, serum (1 ml) derived from wild-type mice were injected at day 8 into wild-type and B-cell-deficiency mice that had been inoculated with LL2 cells (10^6) at day 0. The mice were injected intravenously with 2×10^6 cfu of *Salmonella* at day 8. The mice in each group were sacrificed, and the numbers of *Salmonella* in the tumors, livers, and spleens were determined on LB plates at day 9.

Detection of cytokines expression

To determine the expression of cytokines after *Salmonella* 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. The levels of interleukin-6 (IL-6), tumor necrosis factor (TNF)- α , and interferon (IFN)-γ in the sera, livers, spleens and tumors after *Salmonella* administration were determined by enzyme-linked immunoassay (ELISA) (R＆D, Minneapolis, MN). The protein content in each sample was determined by bicinchoninic acid (BCA) protein assay (Pierce Biotechnology, Rockford, IL).

Infection of tumor cells with *Salmonella*

Tumor cells (10^5/well) that have been cultured in 24-well plates were infected with *Salmonella* (10^6 cfu) or *Salmonella* that had been admixed with various sera collected from mice and incubated for 8 h. The medium was then removed, washed, and replenished with fresh medium supplemented with 50 μg/ml gentamicin. Briefly, supernatants were removed after a further 90 minutes culture with gentamicin, and adherent cells were lysed to release the intracellular bacteria. Lysates were serially diluted in PBS and spread on LB agar plates, and cfu were counted after overnight.

Detection of antibody productions in response to bacteria

Antibodies against bacteria in mice were determined by ELISA. Sera or the extract of tumor were collected from the wild-type and B-cell-deficient mice and examined for the levels of anti-bacterial antibodies as previous described (Lee et al. 2009; Shiau et al. 2005). Antibodies against *Salmonella*, including immunoglobulin G (IgG), and immunoglobulin M (IgM) in mice were determined by the indirect antibody ELISA. In brief, 96-well microtiter plates (Nunc-immuno MaxiSorp plate, Nunc, Roskilde, Denmark) were coated with 100 μl/well of heat-killed *Salmonella* harvested from cultured broth (2×10^8 cfu/ml) in carbonate/bicarbonate coating buffer (pH 9.6) at 4℃ overnight and the free binding site was blocked with BSA diluent/blocking solution (KPL, Baithersburg, MD). After washing five times with PBS containing 0.1% Tween 20, 100μl of the mouse serum or extract were added to each well in triplicate and left for 1 h at 37 ◦C. After washing, 100 μ l of peroxidase-conjugated goat anti-mouse IgG or IgM antibody (KPL) diluted 1:5000 in BSA diluent/blocking solution was added to each well and the plates were incubated at 37 ℃ for 1 h. The plates were washed and developed with 3,3',5,5'-tetramethyl benzidine (KPL). The enzyme reaction was stopped after 30 min incubation at room temperature with 2N H_2SO_4 and the absorption was measured at 450 nm.

Statistical analysis

The unpaired, two-tailed Student's t test was used to determine differences between groups for the comparisons of tumor volume and the numbers of *Salmonella.* The survival analysis was performed using the Kaplan-Meier survival curve and log-rank test. Any *P* value less than 0.05 is regarded statistically significant.

Results

Susceptibility of B-cell-deficient mice to *Salmonella* infection

The clearance of *Salmonella* from infected tissues is controlled by the acquisition of adaptive immunity. The mechanisms of immunity to *Salmonella* were studied by using various immunocompromised mice. $CD4^+$-, $CD8^+$-, B-cell-deficient mice, and wild-type mice treated intravenously with *Salmonella*. Because inoculation of mice with *Salmonella* was shown to result in loss of body weight, we measured their body weight. As shown in Fig. 1a, the body weight of B-cell-deficient mice treated with *Salmonella* had 21% less compared with $CD4^+$-, $CD8^+$-cell-deficient or wild-type mice treated with *Salmonella*. In $CD4^+$-, $CD8^+$-cell-deficient mice and wild-type mice, *Salmonella* did not influence the health of mice. The survival of B-cell-deficient mice was dramatically decreased after *Salmonella* administration (Fig. 1b). Wild-type, $CD4^+$-, and $CD8^+$-cell-deficient mice surviving the infection were recovered at day 7 after *Salmonella* administration. Thus, B-cells are required for control of the virulence of *Salmonella* in host.

Tissue distributions of *Salmonella* in mice

Administration of *Salmonella* influences the health of B-cell-deficient mice by measuring the body weight and the survival. We want to investigate the impact of B-cell on the tissue distributions of *Salmonella* while all the mice were still alive. The

wild-type and B-cell-deficient mice bearing tumors were injected with *Salmonella* and the amounts of *Salmonella* in the tumors, livers, and spleens were determined at various time points. Because all the body weight of mice was decreased at day 1 after *Salmonella* treatment, the result pointed out that *Salmonella* significantly influences the health of mice at this time point. Furthermore, B-cell-deficient mice die from 8-10 days after inoculation with *Salmonella* and the body weight of wild-type mice were recovered at day 7. The health of mice could be clearly identified by choosing the two time points. As shown in Fig. 2, the bacterial amount was higher in the tumors than other healthy organs at day1 and day 7 after *Salmonella* treatment examined in the both strain mice in a separate experiment. Notably, bacterial load in the blood and healthy organs was significantly reduced in the wild-type mice at day 7, and there was still detectable the bacteria in the blood of B-cell-deficient mice (Fig. 2). By measuring the amounts of *Salmonella* in different organs and blood, we demonstrated that *Salmonella* significantly influences the health of B-cell-deficient mice. Our studies suggest that B-cells play important roles in controlling the distribution of *Salmonella*. To rule out the possibility that the data obtained above could be the result of general phenotype rather than mice strain specificity, we used B-cell-depletive antibody to demonstrate the role of B-cells in the control of *Salmonella* distribution. B-cells were depleted from the mice by intraperitoneal injection of anti-B220

antibodies at day 0. The depletion of B-cells was transient. A treatment with anti-B220 antibodies was sufficient to reduce the number of B-cells in to 2% of the number found in untreated control mice at day 4. The percentage of B-cells in the spleen of depleted mice was approaching normal levels on day 8 (Fig. 3a). At time points after depletion, B-cell-depletive mice were sacrificed at 24 h after *Salmonella* treatment and the bacterial amounts were determined (Fig. 3b). The depletion of B-cells in mice contributed to a high colonization of *Salmonella* in all tissues that was 10~1000 times higher than that in non-depleted mice. The B-cell depletion resulted in higher the colonization of *Salmonella* in the tumor sites, but at the same time, the bacterial amounts were increased in the healthy tissues. However, the transient depletion B-cell did not significantly influences the health of mice (Fig. 3c). Thus, these results indicate the B-cells are required for control of the distribution of *Salmonella* in host. Twenty days after tumor inoculation, wild-type mice receiving *Salmonella* had small tumor volume than the control mice receiving PBS (22.94%, $P < 0.01$). Furthermore, tumor growth was significantly retarded in B-cells-depleted mice after *Salmonella* administration (Fig. 3d). Fig. 3d also illustrated that the tumor growth of wild-type mice has the same trend as B-cell depletion. The B-cell depletion without *Salmonella* administration had no effect on tumor growth. The antitumor activities of tumor-targeting *Salmonella* can be amplified by the transient removal of

B-cells in host.

Containment of *Salmonella* in tumor by B-cells

Previous studies and our experiments suggest that B-cells are required for control of the distribution of *Salmonella* in host (Mittrücker and Kaufmann 2000; Mittrücker et al. 2000). Herein, to investigate the impact of B-cell on the distribution of *Salmonella* from tumor sites, wild-type and B-cell-deficient mice bearing tumors were intratumorally injected with *Salmonella*, and the amounts of accumulated *Salmonella* in the tumors, livers, spleens, and blood were determined at various time points (Fig. 4). When *Salmonella* was injected into the tumor, *Salmonella* was found not only in the treated tumors but also in the healthy organs at 7 days after *Salmonella* treatment in both strains of mice. The bacterial numbers in the livers and spleens and the number of bacteria in the blood decreased to undetectable levels in wild-type mice. Bacterial load in the blood was reduced by approximately 1.3 orders of magnitude in wild-type mice at day 7, whereas there was no equivalent decrease in the bacterial number in B-cell-deficient mice. In contrast, bacterial levels in the three tissues increased in B-cell-deficient mice. This suggests that B-cells inhibited the dissemination of *Salmonella* from tumor sites to healthy organs. The B-cells are able to function as a border that inhibits *Salmonella* dissemination from tumor into healthy organs.

Effects of *Salmonella* on cytokines induction in B-cell-deficient mice

The ability of *Salmonella* to survive and replicate in host is dependent on the activation state of the host cells that are affected by host cytokines (Fahy et al. 2004). To determine the role of cytokines in the susceptibility of B-cell-deficient mice to *Salmonella* infection, we determined the plasma levels of proinflammatory cytokines (IL-6 and TNF-α) and IFN-γ, needed for expression of full acquired resistance to *Salmonella* (Mittrücker and Kaufmann 2000). The expressions of inflammation cytokines, including IL-6 and TNF- α, in sera, tumors livers and spleens were measured. Compared with mice received PBS, the levels of IL-6 and TNF-α were significantly increased in sera, livers, spleens and tumors derived from mice treated with *Salmonella* (Fig. 5a, b). Notably, the inductions of inflammation cytokine in B-cell-deficient mice treated with *Salmonella* were increased 2~29-fold compared with control treatment. IFN-γ is the prototype T cell helper 1 (Th1) cytokine and plays a central role in the control of *Salmonella* infection and *Salmonella*-mediated antitumor activities. The level of IFN-γ was not significantly changed in the sera, spleens, and tumors derived from B-cell-deficient and wild-type mice treated with PBS. Compared with wild-type mice that received PBS, the levels of IFN-γ were significantly increased in the sera, spleens, and tumors derived from wild-type mice treated with *Salmonella*. However, IFN-γ production induced by the bacteria was not

significantly observed in B-cell-deficient mice (Fig. 5c). Collectively, these results show that B-cells play a role in the control of expression of inflammatory cytokines and Th1 response after *Salmonella* infection.

The role of natural antibodies in *Salmonella* dissemination

Evidence from human and animal studies suggests that severe infection and bacteremia occur when specific antibody is lack (Gil-Cruz et al. 2009). Nature antibodies are mostly of the IgM isotype, and can bind to a particular antigen or pathogen, even if the host has never exposed to it. To examine whether the anti-*Salmonella* natural antibodies inhibited the invasion of *Salmonella*, the intracellular bacteria were assayed in a gentamicin protection assay. As shown in Fig. 6a, the sera collected from the *Salmonella*-immunized mice inhibited the invasion of *Salmonella* to the cells and the sera collected from the wild-type mice also had the ability to inhibit the invasive ability of *Salmonella* compared with PBS control. The sera from B-cell-deficient mice did not inhibit the invasion of *Salmonella*. Meanwhile, the sera from wild–type and B-cell-deficient mice assessed by ELISA on *Salmonella* revealed *Salmonella*-specific natural IgM, but not IgG, antibodies were detectable up to a dilution of 1:8 (Fig. 6b). The level of anti-*Salmonella* IgM antibodies in tumor derived from mice treated with *Salmonella* was higher than that derived from mice treated with PBS (Fig. 6c). We next determined whether natural antibodies could

decrease *Salmonella* dissemination to healthy organs in B-cell-deficient mice by adoptive transfer of sera from wild-type mice into B-cell-deficient recipient mice. Sera harvested from wild-type mice were injected intravenously into the wild-type or B-cell-deficient recipients. B-cell-deficient mice to which had adoptively transferred sera from wild type mice, had reduced the bacterial load in all tissues, as compared with those receiving PBS (Fig. 6d). These experiments suggest that the high susceptibility of B-cell-deficient mice to *Salmonella* administration is associated with a failure to control *Salmonella* dissemination in tumors.

DISCUSSION

In the studies reported here, an important opportunity is provided by our animal models to monitor the evolution of the B-cell mediated immunity to tumor-targeting *Salmonella* in tumor-bearing mice. Our studies were performed in the wild-type and B-cell-deficient mice to unravel a possible role between antibodies and tumor-targeting *Salmonella*.

The host immunoresponse after administration by *Salmonella* is highly complex. It is not only restricted to the myeloid cells but also lymphoid cells. Mice containing defects that affect immunity are incapable of clearing infection with *Salmonella*. These include T-cell receptor αβ, major histocompatibility complex class II, interferon (IFN) γ, and Igh knockouts, all of which are susceptible to *Salmonella* infection (McSorley and Jenkins 2000). Experiments with gene-deficient mice demonstrated that control of primary infection with *Salmonella* depends on $CD4^+$ T-cells and B-cells also play a distinct role in control of *Salmonella* in mice (Mittrucker et al. 2000).The effective clearance of a primary infection with attenuated *Salmonella* is dependent on costimulatory signals to T-cells via CD28 but is completely independent of B-cells (McSorley and Jenkins 2000). In contrast, some studies evaluated that the role of B-cells in long-term acquired immunity to virulent *Salmonella* in mice vaccinated with attenuated *Salmonella* strains (Mastroeni et al.

2000). In our previous works, the specific anti-*Salmonella* antibodies interfere with the invasion and gene transfer of *Salmonella* (Lee et al. 2009). The specificity and titer of antibody play important roles in the regulation of the tumor-targeting potential of *Salmonella*. Therefore, our previous data, combined with those reported previously (Westphal et al 2008; Lee et al. 2008a; Lee et al. 2004; Lee et al. 2011), suggest that the abilities of *Salmonella* in initial localization and subsequent replication within tumors are not fully dependent on the host's innate 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. The humoral immunity of naïve host consists of B-cells secreting natural antibodies representing the spontaneous repertoire of circulating immunoglobulins that belong to IgM class (Ochsenbein et al. 1999). A major source of natural antibodies seems to the B-1-cell subset. There is also evidence for natural antibodies with specificities for a wild range of bacterial antigens (Reid et al. 1997). Dependent on other and our studies, bacteremia occur when antibodies is lacking. Although generation of antibodies is a central function of B-cells, they can perform alternative functions such as antigen presentation, initiation of T-cell response and cytokine production. Cross-talk among B-cells and T-cell is necessary for T-cell

function and elevated expression of co-stimulatory molecules on B-cells may contribute to the proliferation and activation of T-cells. Meanwhile, we demonstrate that *Salmonella* induced IFN-γ production and polarized the T-cell response to a Th1-dominant state in wild-type mice, but not in B-cell-deficient mice. B-cell also drive IFN- γ production from T-cell (Barr et al. 2009). Our results prove that B-cells in host are necessary for using the therapy of tumor-targeting *Salmonella*.

The tumor targeting efficiency was determined to be *Salmonella* doses; administration routes; tumor volumes; the distance from tumors to injected sites (Mei et al. 2002). The factors that account for efficient the antitumor effect of *Salmonella* is highly complex. 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 migration of *Salmonella* in the tumor sites and the antitumor effect of *Salmonella* are restricted by neutrophils (Westphal et al. 2008). In our studies, host B-cells not only take part in the tumor colonization of *Salmonella* but also play a role in the control of *Salmonella* dissemination. Our results show that the B-cells restrict the dissemination of *Salmonella* spread from tumor sites to healthy organs (Fig. 4) and anti-*Salmonella* IgM antibodies exist in tumor microenvironment (Fig 6c). B-cells may regulate in the induction of T cell responses in the early phase of *Salmonella* infection and secreted antibody against *Salmonella*. In the work

described here, natural antibodies produced by B-cells result in a slightly lower total number of bacteria in the tumor sites, but decrease the inflammation and cytokine production after systemically *Salmonella* treatment. Importantly, when avoiding natural antibodies produced by B-cells by B-cell depletion at the time of infection or using B-cell-deficient mice, the higher bacterial loads were found in healthy organs. This may be accompanied by a higher number of bacteria in the healthy organs of host, a strongly increased formation of sepsis, and decreased the specificity of tumor-targeting *Salmonella*. Although the B-cell depletion in host resulted in the dissemination of *Salmonella* to healthy organs, the therapeutic results were promising. Avoiding side effect emerges as a key issue in the development of safety *Salmonella* therapy approaches. These results will develop the new opinion that gives thought to increase the safety therapeutic potential of tumor-targeting *Salmonella*.

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Disclosure Statement

The authors have no conflict of interest.

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Figure legends

Fig. 1.

Susceptibility of B-cell-deficient mice to infection with *Salmonella* (S.C.). (a) The B-cell, $CD8^+$, and $CD4^+$-deficient mice and wild-type mice were injected intravenously with *Salmonella* (2×10^6 cfu) on day 0 and the body weights of mice were determined (means ± SD, n = 6). $P < 0.001$ for B-cell-deficient mice *versus* Wild-type mice, CD4+-cell-deficient, or CD8+-cell-deficient mice (b) Kaplan–Meier survival curves on day 16 are shown. (mean ± SD, n=6) These experiments were repeated with similar results. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Fig. 2.

The spatial and temporal distribution of *Salmonella* (S.C.) in B-cell-deficient and wild type mice after intravenous administration. The mice bearing tumors ranging from 50 to100 mm^3 were injected intravenously with *Salmonella* (2×10^6 cfu), and the amounts of *Salmonella* in the blood, tumors, livers, and spleens were determined at day 1 and day 7 (mean ± SD, n = 3-4) postinfection. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Fig. 3.

Effect of B-cell depletion on tumor-targeting *Salmonella.*

(a) B-cells were depleted by injection of anti-B220 into mice. At various time points, spleen was collected and analyzed by flow cytometry for the presence of B-cells. (b) The effect of B-cell depletion on the colonization of *Salmonella*. The tumor-bearing mice were injected intraperitoneally with anti-mouse B220 antibodies on day 0. On days 1, 3, 5, 7 after depletion, the mice were intravenously injected with *Salmonella* (2×10^6 cfu), respectively. The amounts of *Salmonella* in the tumors, livers, spleens, and blood were determined after 24 h (mean ± SD, n = 3-4) postinfection. (c) The tumor-bearing mice were injected intraperitoneally with anti-mouse B220 antibodies at day 8. The B-cell-depleted and wild-type mice were injected intravenously with *Salmonella* (2×10^6 cfu) at day 8 and the body weights of mice were determined (means ± SD, n = 6). (d) Comparison of tumor growth in B-cell-depleted and wild-type mice after *Salmonella* administration. Groups of 8 mice that had been inoculated with LL2 cells (10^6) at day 0 were depleted with anti-B220 antibodies at day 8 followed by *Salmonella* (2×10^6 cfu) at day 8. Vehicle control mice received PBS. Tumor volumes (mean ± SEM, n = 8) among different treatment groups were compared in mice bearing LL2 ($P < 0.001$ for B-cell-depleted + PBS *versus*; B-cell-depleted + S.C. $P<0.05$ for Wild-type mice +PBS *versus* Wild-type mice +S.C. and for Wild-type mice + S.C. *versus* B-cell-depleted mice +S.C.) These experiments were repeated with similar results.*, $P < 0.05$; **, $P < 0.01$; ***, $P <$

0.001.

Fig. 4.

The spatial and temporal distribution of *Salmonella* (S.C.) in B-cell-deficient and wild-type mice after intratumoral administration. The mice bearing tumors ranging from 50 to100 mm^3 were injected intratumorally with *Salmonella* (2×10^6 cfu), and the amounts of *Salmonella* in the blood, tumors, livers, and spleens were determined at day 1 and day 7 (mean ± SD, n = 3-4) postinfection. This experiment was repeated with similar results. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Fig. 5.

Effects of *Salmonella* (S.C.) on cytokine production in B-cell-deficient and wild-type mice bearing tumors. The mice bearing tumors were injected intravenously with *Salmonella* (2×10^6 cfu). (a) IL-6, (b) TNF-α and (c) IFN-γ levels in the sera, tumors, livers, and spleens were measured by ELISA at 12 h postinfection (mean ± SD, n = 3-4). This experiment was repeated with similar results.

Fig. 6.

Detection of nature antibody production in response to *Salmonella* (S.C.). (a) LL2

cells were infected with *Salmonella* at 10^6 cfu or *Salmonella* admixed with various sera obtained from the mice. Gentamicin protection assay in these cells were examined 9.5 h later. (means ± SD, n=3). (b) Detection of natural antibodies in sera. Sera from mice were tested in an ELISA on *Salmonella* coated plates for the presence of specific IgM or IgG. (c) Detection of natural antibodies in tumor extract. Tumor extract from mice were tested in an ELISA on *Salmonella* coated plates for the presence of specific IgM. (d) Nature antibodies influence the distribution of *Salmonella*. Groups of 4 wild-type and Bell-deficient mice that had been inoculated with 10^6 LL2cells at day 0 were adoptively transferred with sera (1 ml) from wild-type mice at day 8. The tumor bearing mice were injected with intravenously *Salmonella* (2×10^6 cfu) at day 8 and the amounts of *Salmonella* in the blood, tumors, livers, and spleens were determined at day 9 (mean ± SD, n = 4). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.