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An Enteric Pathogen *Salmonella enterica* serovar Typhimurium Suppresses Tumor Growth by Down Regulating CD44^{high} and CD4 T Regulatory (T_{reg}) Cell Expression in Mice: Critical Role of Lipopolysaccharide and Braun Lipoprotein in Modulating Tumor Growth

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Abstract

An anti-tumor activity associated with several bacterial pathogens, including *Salmonella enterica serovar* Typhimurium, has been reported; however, the underlying immunological mechanism(s) that leads to an anti-tumor effect is currently unclear. Further, such pathogens cannot be used to suppress tumor growth because of their potential for causing sepsis. Recently, we reported the characterization of *S*. Typhimurium isogenic mutants from which the Braun lipoprotein genes (*lppA* and *B*) and the multicopy repressor of high temperature requirement (*msbB*) gene were deleted. In a mouse infection model, two mutants, namely, *lppB/msbB* and *lppAB/msbB*, minimally induced proinflammatory cytokine production at high doses and were non-lethal to animals. We demonstrated that immunization of mice with these mutants, followed by challenge with the wild-type *S*. Typhimurium could significantly suppress tumor growth, as evidenced by an 88% regression in tumor size in *lppB/msbB* mutant-immunized animals over a 24-day period. However, the *lppAB/msbB* mutant alone was not effective in modulating tumor growth in mice, while the *lppB/msbB* mutant alone caused marginal regression in tumor size. Importantly, we demonstrated that CD44$^+$ cells grew much faster than CD44$^-$ cells from human liver tumors in mice, leading us to examine the possibility that *S.* Typhimurium might down-regulate CD44 in tumors and splenocytes of mice. Consequently, we found in *S*. Typhimurium-infected mice that tumor size regression could indeed be related to the down-regulation of CD44^{high} and CD4$^+$CD25$^+$ T_{reg} cells. Importantly, the role of lipopolysaccharide and Braun lipoprotein was critical in *S*. Typhimurium induced-anti-tumor immune responses. Taken together, we have defined new immune mechanisms leading to tumor suppression in mice by *S*. Typhimurium.

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Disclosures

The authors have no financial conflict of interest.

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Keywords

Salmonella Typhimurium and its mutants; CD44; T regulatory (T_{reg}) cells; tumor; mouse model

Introduction

Salmonella enterica serovar Typhimurium has been shown to possess anti-tumor activity in a mouse model, the capability to selectively amplify within tumors, and express therapeutic proteins 1, 2. Reports have indicated that *S*. Typhimurium selectively infects and preferentially colonizes solid tumors of cancer patients 3–5, making it potentially useful as a vehicle to target human tumors in *vivo* 1, 2, 6–8. Further, *S.* Typhimurium has a significant ability to infect non-phagocytic cells via expression of a type-III secretion system (T3SS), which facilitates bacterial penetration of host cells 9.

S. Typhimurium releases lipopolysaccharide (LPS) during both *in vitro* and *in vivo* growth 10, and its release is significantly enhanced during bacterial lysis following exposure to antibiotics or human serum. This enhanced LPS release causes septic shock 11. Likewise, Braun lipoprotein (Lpp) is also a critical bacterial component in the induction and pathogenesis of septic shock. Like LPS, it induces production of tumor necrosis factor (TNF)-α and interleukin (IL)-6 in mouse and human macrophages *ex vivo* 12, 13, and leads to lethal shock as a result of these cytokine production in both LPS-responsive and non-responsive mice 14. More importantly, Lpp synergizes with LPS to induce production of pro-inflammatory cytokines in mice, because Lpp binds to toll-like receptor (TLR)-2, whereas LPS binds to TLR-4 and CD14 to activate host cells 15. The biological potency of LPS can be significantly reduced when the multicopy repressor of a high temperature requirement (*msbB*) gene is deleted from *S*. Typhimurium 2. This gene codes for an enzyme which is responsible of adding myristic acid to the lipid A moiety of LPS 16. Consequently, in our recent studies, we characterized isogenic mutants of *S*. Typhimurium that were deleted for the Braun lipoprotein (*lppA* and *B*) genes in conjunction with the *msbB* gene 16. We provided evidence that such mutants were highly attenuated in a mouse model of salmonellosis and produced minimal levels of pro-inflammatory cytokines and chemokines 17. We predicted that the *lpp/msbB* mutants of *S*. Typhimurium would be excellent live-attenuated vaccine candidates. Indeed, mice immunized with such mutants were solidly protected against challenge with lethal doses of wild-type (WT) *S*. Typhimurium, in that the *lppB/msbB* and *lppAB/msbB* mutants provided maximum protection. These two mutants were further characterized in terms of their immune responses in a mouse model 17 and examined in this study for their anti-tumor activity.

$CD4^{+}CD25^{+}$ T regulatory (T_{reg}) cells have been shown to be controlling self-reactive T cells by helping to maintain immunological self-tolerance 18, 19 and hence are a major obstacle in effective anti-tumor immunotherapy 20, 21. $CD4^{+}CD25^{+}$ T_{reg} cells have been reported to be specifically recruited to tumor sites and to effectively block anti-tumor cytotoxic T-lymphocyte (CTL) responses. Hence, the targeted removal or inactivation of the $CD4^{+}CD25^{+}$ T_{reg} cells in animal models could lead to improved tumor immuno-surveillance, better vaccine efficacy, and enhanced anti-tumor immunity 22–24. Further,

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CD44 is the principal receptor of the hyaladherin receptor family, and CD44-hyaluronan interactions mediate cell adhesion and migration in various physiological and patho-physiological processes 25–28. CD44 is expressed on many tissues in developing and adult humans, and it was described originally as a homing receptor required for binding of lymphocytes to high endothelial venules 29, 30 . CD44 has also been shown to be involved in lymphocyte activation in humans 31, 32and in a mouse model 33. CD44 expression by tumor cells can increase their interaction with endothelial cells and transmigration across an endothelial monolayer. Several reports have shown that CD44 inhibitors, including anti-CD44 monoclonal antibody and hyaladherin inhibitors, can be used to block tumor cell growth, invasion, and metastasis 34, 35.

In this study, we showed that *lpp/msbB* mutants alone were not effective in suppressing tumor growth in mice. However, we observed a significant regression in the size of tumors implanted in mice first immunized with the *lpp/msbB* mutants of *S.* Typhimurium and then challenged with WT *S*. Typhimurium. Further, we noted a decrease in $CD4^{+}CD25^{+}$ T_{reg} cell numbers and down-expression of $CD44^{high}$ in the spleen of mice infected with *S*. Typhimurium, which for the most part could be mediated through LPS and Lpp.

Materials and Methods

Bacterial cultures

The WT *S.* Typhimurium 14028 strain and its various mutants used in this study are listed in Table 1. The organisms were grown in Luria-Bertani (LB) broth or on LB agar plates in the presence of the appropriate antibiotics. For the growth of *msbB* mutants, we used a special MsbB medium as previously described 17. The MsbB medium/liter consisted of the following: 10 g tryptone, 5 g Yeast Extract, 1 ml 1 M $MgSO_4$, and 1 ml 1 M $CaCl_2$. The bacteria were cultivated at 37°C overnight with shaking at 200 rpm, harvested by centrifugation (6,000 rpm for 5 min), washed with phosphate-buffered saline (PBS), and resuspended in a minimal amount of PBS. Bacteria were counted by determining colony-forming units (cfu) in triplicate, and expressed as cfu/ml.

Animals

We used 6- to 8-week-old C57BL/6, BALB/c, and/or Swiss-Webster female mice (Taconic Farms, Germantown, NY). For selected studies, we used breeding pairs of IL-$10^{-/-}$ mice in a C57BL/6 background (The Jackson Laboratory, Bar Harbor, ME), and IL-$10^{-/-}$ aged mice (2 years old). We used IL-$10^{+/+}$ and IL-$10^{-/-}$ mice as IL-10 is a pleiotropic cytokine with anti-inflammatory and anti-angiogenic properties in *in vitro* and *in vivo* models 36, 37. High IL-10 levels result in smaller tumors, their lesser metastases and reduced angiogenesis in humans, and low IL-10 production is associated with increased risk of prostate cancer 38. Mice were kept under specific pathogen-free conditions in filter-topped cages with sterile bedding and fed sterile food and water.

Infection/immunization of mice

Mice were infected/immunized *via* the intraperitoneal (i.p.) route with: WT *S.* Typhimurium14028, *lppB/msbB* mutant, and/or the *lppAB/msbB* mutant (Table 1) at doses

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ranging from 0.5×10^2–2×10^3 cfu/100 μl, and deaths were recorded for a 30-day period. In some cases, mice were initially immunized with the mutant *S*. Typhimurium strains and then rechallenged with the WT *S*. Typhimurium strain (2×10^3 cfu) 30 days after the initial immunization. The animal experiments were performed under the approved Institutional Animal Care and Use Committee and death was the end point of the study when animals were infected with the WT bacteria. However, those animals that were immunized with the mutant *S*. Typhimurium strains and then challenged with the WT bacterium survived and were humanely euthanized after 30 days.

Bacterial counts

At indicated time intervals following the infection/immunization of mice, spleens were removed and homogenized in PBS, and serial dilutions of the homogenates were plated on LB and *Salmonella-Shigella* agar plates to determine bacterial counts. The plates were incubated at 37°C for 24 to 48 h and colonies counted.

Tumor cells

The B16F1 melanoma cell line from ATCC (American Type Culture Collection, Manassas, VA) was cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM of L-glutamine before injections into mice under an approved animal protocol. These cells were grown for 6 days at 37°C in the presence of 5% CO_2 before injections.

Human liver cancer cells (from the Sealy Cancer Center, University of Texas Medical Branch, Galveston, TX) were stained with mouse anti human CD44 PE antibody (PE, clone 515) (BD Bioscience, Franklin Lakes, NJ). After washing with PBS, $CD44^+$ and $CD44^-$ tumor cells were isolated by using a FACSAria (BD Bioscience). Each cell clone population was expanded in DMEM supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM of L-glutamine in 96-well tissue culture microtiter U-bottom plates.

Tumor cell injections

Six- to eight- week-old female C57BL/6 mice were subcutaneously (s.c.) injected with tumor cells 5×10^5/mouse/100 μl. Personnel blinded to the study design measured tumor growth by microcaliper 3 times a week. Tumor volume was calculated according to the formula: (length)×(width)×(width)/2.

Flow cytometric analysis

Spleen cells were obtained from mice infected with WT or mutant strains of *S*. Typhimurium. Splenocytes (10^5) were suspended in PBS and 1% FBS, and incubated with mouse CD16/CD32 monoclonal antibodies (0.25 μg/100 μl) (BD Bioscience) for 15 min at room temperature. These antibodies were added to block antibody binding to mouse Fc-γ receptor-bearing cells. Then, the cells were washed twice with PBS plus 1% FBS. To quantitatite T cells, the cell suspension was incubated for 60 min at 4°C with anti-CD4 (BD Bioscience, clone GK1.5) conjugated to fluorescein isothiocyanate [FITC] (0.25 μg/100 μl), anti-CD44 (BD Bioscience, clone IM7) antibody conjugated to phycoerythrin (PE) (0.1 μg/100 μl), and anti-CD25 (BD Bioscience, clone 3C7) antibody conjugated to anti-

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allophycocyanin (APC) (0.1 μg/100 μl). Subsequent to incubation, the cells were washed twice with PBS and then analyzed by FACScan flow cytometry using CellQuest (Becton Dickinson, Mountain View, CA) software. The corresponding isotype controls (rat IgG1, IgG2a, and IgG2b) were purchased from eBioscience (San Diego, CA) and BD Bioscience, respectively.

Statistics

At least three independent experiments were performed, and the data were analyzed using Student's t test. P values of 0.05 were considered significant. Statistically significant values are referred to as follows: *, *p<0.05*; **, *p<0.01*; ***, *p<0.001*.

Results

Effect of WT *S.* Typhimurium and its *lpp/msbB* mutants on tumor growth

We opted to immunize mice with various *S*. Typhimurium strains before transplantation of tumors, as we did not intent to develop tumor-antigen-specific immune/memory response, which could potentially interfere with *S.* Typhimurium-induced strong cellular and humoral immune responses. Further, as discussed in the next section, it was important to immunize animals with the mutant *S*. Typhimurium strains as we could use high doses of them without inducing any lethality and then challenge mice with a high dose of WT bacteria, which otherwise will be lethal to animals in an un-immunized group. Finally, people traveling from the developed world to developing countries are invariably vaccinated with the live-attenuated *Salmonella* strain and therefore it appeared logical to immunize animals with *S.* Typhimurium .

To delineate whether the WT *S.* Typhimurium or its mutant strains suppressed the growth of melanomas, mice were infected with 50 cfu/mouse of either the WT *S*. Typhimurium or its *lppAB/msbB* or *lppB/msbB* mutant strain. Mice injected with PBS served as a negative control. After 30 days, B16 cells (5×10^5) were subcutaneously injected into mice. The mean tumor size for various bacterial strains after 24 days was: WT S. Typhumirum, 640 +/−52 mm^3; *lppB/msbB* mutant, 798 +/− 65 mm^3; *lppAB/msbB* mutant, 871+/−62 mm^3; and PBS, 912+/−74 mm^3 (Figure 1A). As can be noted from this figure, tumor size was inhibited in WT *S.* Typhimurium-infected mice by a statistically significant 30% compared to the rate in animals that were given PBS alone. The decreased tumor size was only marginal (12%), however, in mice infected with the *lppB/msbB* mutant after 24 days, and non-existent for up to 21 days in animals infected with the *lppAB/msbB* mutant when compared to the PBS controls. A slight, but statistically insignificant decrease in tumor size occurred after 24 days with the *lppAB/msbB* mutant (Figure 1A).

Consequently, we further investigated, using a different approach, the anti-tumor effects of WT *S*. Typhimurium and its mutants. In this set of experiments, mice were immunized with the *lppAB/msbB* and *lppB/msbB* mutants of *S*. Typhimurium at a dose of 2×10^3 cfu. Our previous studies indicated that mice were fully protected against mortality by i.p. doses of up to 1×10^6–1×10^7 cfu of these mutants and demonstrated that animals immunized with the above mutants at doses of 1×10^6– 1×10^7 cfu were 100% protected against similar

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doses of the WT bacteria after rechallenge 17. After 30 days of immunization of these mice with the above mutants, we rechallenged them with the WT *S*. Typhimurium at a dose of 2×10^3 cfu. Two weeks after rechallenge of mice with the WT bacteria, B16 cells (5×10^5) were implanted s.c. into the animals. The mean tumor sizes in mice immunized with the mutants, followed by WT infection, were as follows: *lppAB/msbB* mutant alone, 863+/−71 mm^3; *lppB/msbB* mutant alone, 660+/−49 mm^3, *lppAB/msbB* + WT, 470+/−31 mm^3, and *lppB/msbB* + WT, 110+/−9 mm^3 (Figure 1B). The tumor size in mice injected with PBS alone was 903+/−86 mm^3 and served as a positive control. It is evident from these data that tumor size regressed in the animals first immunized with the mutants and then challenged with the WT *S*. Typhimurium, and that *lppAB/msbB* and *lppB/msbB* mutants, respectively, provided 48% and 88% protection. The protection afforded by the *lppB/msbB* and *lppAB/msbB* mutants alone at a dose of 2×10^3 cfu was 27% and 5%, respectively (Figure 1B).

Infection of mice with WT *S.* Typhimurium and its *lppB/msbB* mutant down-regulated expression of $CD4^+CD25^+$ T_{reg} cells

To define the underlying mechanism(s) involved in S. Typhimurium-induced tumor growth suppression, we infected Swiss-Webster mice with WT *S.* Typhimurium or its *lppAB/msbB* and *lppB/msbB* mutants (1×10^3cfu/mouse). After 5 days of infection, spleens from infected mice were stained with anti-CD4 FITC and anti-CD25 APC, and the splenocytes analyzed by flow cytometry. We found that the $CD4^+CD25^+$ T_{reg} cells decreased significantly in the WT group of infected mice (0.5%) compared to uninfected animals (1.6%, a decrease of 69%) (Figure 2A). The number of $CD4^+CD25^+$ T_{reg} cells decreased slightly by 31% in mice infected with the *lppB/msbB* mutant (1.1% versus 1.6% for control), and minimally affected (1.5%) in the *lppAB/msbB*-infected mice compared to the controls (1.6%). The percentage of $CD4^+$ cells were slightly decreased in animals infected with the WT *S*. Typhimurium (18%) when compared to animals infected with either the *lppB/msbB* (21%) or the *lppAB/msbB* (22%) mutant and/or those mice that were only given PBS (24%). In addition we found that the expression of gene-encoding Forkhead box P3 (Foxp3), which controls T_{reg} cell development and their functions 39, was down-regulated in the splenocytes of mice infected with WT *S*. Typhimurium (0.6%), when compared to splenocytes of control uninfected mice (1.8%). As a positive control, we used splenocytes treated with *S*. Typhimurium lipopolysaccharide (3 μg/ml), which, as expected, decreased the expression of the gene-encoding Foxp3 significantly (0.31%) compared to the controls (Figure 2B). Some down-regulation of T cell activation (20%) was also seen in splenocytes infected with the WT *S*. Typhimurium *ex vivo*.

Immunization of mice with the *lppAB/msbB* mutant followed by challenge with the WT *S.* Typhimurium increased the number of CD4, $CD4^+CD25^+T_{reg}$, natural killer (NK) cells as well as macrophages in the tumor of mice

An important question which should be considered is whether there are increases in T- and/or NK cell lytic activity against B16 melanoma cells in mice which were first immunized with the *S*. Typhimurium mutant (e.g., *lppAB/msbB*) and then challenged with the WT *S*. Typhimurium compared to those mice that were immunized with the mutant but challenged with the same *lppAB/msbB* mutant. The above-mentioned scenarios would lead to reduced tumor growth. Therefore, we immunized C57BL/6 mice with the *lppAB/msbB*

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mutant at a dose of 2×10^3 cfu. After 30 days, we challenged them with the WT *S.* Typhimurium at a similar dose of 2×10^3 cfu. After two weeks, B16 cells (5×10^5) were implanted s.c. into the animals, and three weeks later, tumor cells from these mice were stained with CD4-PE-Cy7, CD25-APC, NK1.1-FITC, CD11b-PE, CD3-PE, interferon (IFN)-γ–FITC antibody and analyzed by flow cytometry. Our data indicated that the number of CD4, CD4+CD25+, and NK cells as well as macrophages were increased to 1.8%, 3.2%, 2.6%, and 7%, respectively, in tumors of mice that were first immunized with the *lppAB/msbB* mutant before WT *S.* Typhimurium challenge. These numbers were in contrast to 0.1%, 0.4%, 0.5%, and 1.5% in those animals that were immunized with the mutant and then challenged with the mutant. Likewise, animals that were given only PBS had the following percentages of CD4, CD4+CD25+, NK, and macrophages: 0.1%, 0.3%, 0.2%, and 1.6%, respectively. Importantly, the percentage of IFN-γ producing T cells that infiltrated into tumor of mice that were first immunized with the *lppAB/msbB* mutant and then challenged with the WT *S*. Typhimurium was also much higher compared to in tumors of mice that were immunized with the *lppAB/msbB* mutant and subsequently challenged with the mutant. Animals given only the PBS but injected with B16 cells had baseline level of IFN-γ (Figure 3A–C).

WT and *lpp/msbB* mutants of *S.* Typhimurium down regulated the expression of CD44 in mouse melanoma B16 cells *in vitro* and in the spleens of mice *in vivo*

We cultured B16 cells with WT *S.* Typhimurium or its *lppAB/msbB* and *lppB/msbB* mutants at a multiplicity of infection of 1 in DMEM at 37°C in the presence of 5% CO_2. After 24 h of incubation, the cells were harvested and stained with mouse anti-CD44 PE and analyzed by flow cytometry. As noted from Figure 4A, WT *S.* Typhimurium-infected B16 cells exhibited a significant decrease in the expression of CD44 marker (69%) compared to that of control (83%). Likewise, the expression of CD44 showed a downward trend, with decreases to 74% and 71% in B16 cells infected with the *lppAB/msbB* and *lppB/msbB* mutant, respectively, compared to uninfected B16 cells (83%).

We also infected Swiss-Webster mice with WT *S.* Typhimurium or its *lppAB/msbB* and *lppB/msbB* mutants (1×10^3 cfu/mouse). After 5 days of infection, spleen cells were stained with anti-CD44 PE, and analyzed by flow cytometry. As noted from Figure 4B, the splenocytes of WT *S.* Typhimurium-infected group exhibited a significant decrease (3.4% versus 12.6%; indicated by an arrow) in the expression of $CD44^{high}$, followed by the *lppB/msbB* (6.5%) and *lppAB/msbB* (9.7%) mutants compared to the control splenocytes from uninfected mice.

CD4 T_{reg} cells in $CD44^{high}$ cells from the spleens of naive C57BL/6 mice and $IL10^{-/-}$ aged mice

Splenocytes from naive C57BL/6 and $IL10^{-/-}$ aged mice were stained with mouse anti-CD4 FITC, anti-CD25 APC and anti-CD44 PE antibodies and analyzed by flow cytometry. R1 cells were gated as lymphocytes and R1+R2 as $CD44^{high}$ cells, while R1+R3 represented $CD44^{med}$ cells (Figure 5A). $CD44^{high}$ cells contained much higher levels of $CD4^+CD25^+$ T_{reg} cells (1.82%), compared to the levels in the $CD44^{med}$ (1.13%) and control splenocytes (1.61%) of naive C57BL/6 mice (Figure 5B). In $IL\text{-}10^{-/-}$ aged mice, $CD44^{high}$ cells

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contained much higher levels of $CD4^+CD25^+$ T_{reg} cells (2.3%) compared to the levels in $CD44^{med}$ (0.7%) and control splenocytes (1.8%) (Figure 5C).

Higher levels of $CD44^{high}$ expression in the spleens of IL-$10^{-/-}$ aged mice

Splenocytes from C57BL/6 and IL-$10^{-/-}$ aged mice were stained with mouse anti-CD44 PE antibodies. The splenocytes from C57BL/6 aged mice expressed higher levels of CD44 than did those from the young mice (Figure 6A; 2.06% versus 3.97%). The splenocytes from IL-$10^{-/-}$ aged mice expressed even higher levels of CD44 than did the IL-$10^{-/-}$ young mice (Figure 6B; 2.5% versus 13.6%). We then subcutaneously implanted B16 tumor cells in IL-$10^{-/-}$ aged mice. After 24 days, mice with bigger or smaller tumors were killed and their splenocytes stained with mouse anti-CD44 PE antibodies. In general, the mice having the larger tumors expressed higher levels of $CD44^{high}$ (12.5% versus 8.4%) than did animals with smaller tumors (Figure 6C).

Growth of $CD44^+$ and $CD44^-$ cells from human liver tumors in mice

We sorted $CD44^+$ and $CD44^-$ cells from human liver tumors and grew them in DMEM. After expansion of the cell colonies in 96-well microtiter plates, the cells were transferred to 25-cm^2 cell culture flasks. Six days later, the $CD44^+$ and $CD44^-$ tumor cells (5×10^5/100 μl) were injected s.c. into BALB/c mice. The mean tumor size for the $CD44^+$ group was 1075 +/−175 mm^3 compared to 62 +/− 42 mm^3 for the $CD44^-$ group on day 20 after injection (Figure 7). These data indicated that CD44 was required for tumor growth and represented an important tumor marker.

Discussion

The exact mechanism by which *Salmonella* strains result in an inhibition of tumor growth is still unclear. Some earlier studies reported the construction of mutant strains of *S*. Typhimurium, such as *msbB* and auxotrophic mutants that were nonpathogenic for mice, pigs, and humans. These were also demonstrated to have accumulated 1000-fold more in tumors than in other organs. However, the clinical application of such strains did not successfully curtail tumor growth 2, 40.

In this study, we showed that the *lppAB/msbB* mutant did not suppress tumor growth, while the *lppB/msbB* mutant did somewhat slow-down the growth of the tumors compared to those of controls in the 24 days' observation (Figure 1A). Importantly, tumor size in mice immunized with either of the mutants and then infected with the WT bacteria was significantly reduced (Figure 1B). These data indicated that the *S*. Typhimurium mutants that were deleted for the *msbB* and *lpp* genes lost this tumor repressor function, and LPS and Lpp were critical for *S.* Typhimurium's anti-tumor function.

The $CD4^+CD25^+$ T_{reg} cells play a crucial role in tumor immune pathogenesis, and they also modulate immune therapeutic efficacy 41–44. We explored the mechanism(s) underlying an *S.* Typhimurium-mediated, anti-tumor effect, and found that $CD4^+CD25^+$ T_{reg} cells from spleens of mice were significantly decreased (69%) in the WT *S.* Typhimurium-infected mice and decreased by 31% in *lppB/msbB* mutant-infected mice (Figure 2A). This decrease in T_{reg} cell development correlated with the decreased Foxp3 expression in the splenocytes

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of mice infected with the WT *S*. Typhimurium (Figure 2B). LPS has been shown to reduce expression of Foxp3 in lymphocytes 45, 46. Our data presented in Figure 2A and Figure 4B tend to suggest that the expression of CD44^{high} on T cells could in part be related to both T cell activation and T_{reg} cell population. We must emphasize here that for some experiments we preferred to use out-bred Swiss-Webster over in-bred C57BL/6 mice as the latter are more sensitive to *S*. Typhimurium infection, since as little as 50 cfu resulted in the deaths of 30–50% of these mice. Therefore, in those experiments in which higher doses of bacteria were used, we used Swiss-Webster mice. We should cautiously interpret data as different strains of mice might behave differently to *S.* Typhimurium infection as it relates to tumor growth.

S. Typhimurium-induced CD8 CTL and NK cells can kill tumor cells directly 47–49. Likewise, *S.* Typhimurium promotes the maturation of dendritic cells (DCs) and enhances the ability of antigens to present to DCs 50. It has been shown that splenic T cells are broadly activated in the host later during *Salmonella* infection 51. Further, salmonellae-induced IL-12 and IFN-γ are two critical anti-tumor cytokines 52, and IFNγ-producing T cells are significantly elevated after primary infection 53. Our results showed that CD4, $CD4^+CD25^+$, and NK cells as well as macrophages were significantly increased in the tumors of mice that were first immunized with the *lppAB/msbB* mutant and then challenged with the WT *S*. Typhimurium compared to those animals that were only immunized or given PBS (Figure 3). It is known that CD4 and NK cells could inhibit tumor growth 54, 55, but the function of $CD4^+CD25^+$cells in modulating the growth of tumors is not clear. Based on our data, $CD4^+CD25^+$ cells appeared to be recruited into tumors but they might not inhibit tumor growth directly but might prevent suppressor CD4 T cells to enter tumors. We found $CD4^+CD25^+$ cell numbers to be lower in smaller size tumors than in bigger size tumors after infection of mice with the WT *S.* Typhimurium (data no shown). Further, IFN-γ producing cells were significantly increased in tumors that were first immunized with the *lppAB/msbB* mutant and then challenged with the WT *S*. Typhimurium, suggesting that Th1 response might be important for the anti-tumor response.

CD44 has been shown to be highly expressed in many tumor cells 56 such as colorectal cancer stem cells, and expression of CD44 in immortalized cancer cell lines is of functional importance 57, 58. CD44 has recently been shown to be an important marker of cancer stem cells or cancer-initiating cells59, 60. Both CD44 and its variant (vCD44) when expressed in cancer cells, correlate strongly with invasiveness, metastasis, and tumor growth 61, 62. Likewise, $CD44^+$ cancer stem cells have been shown to have enhanced potential for proliferation, migration, and invasion 63. We noted that CD44 was expressed at a significant level in B16 tumor cells. However, its expression was decreased in B16 tumor cells infected with *S*. Typhumirum, albeit to different levels with WT versus the mutants (Figure 4A).

WT *S.* Typhimurium strains used in this study did not only directly decrease CD44 expression of tumor cells in *vitro* but also CD44 expression of lymphocytes in *vivo* (Figure 4B). It has been shown in the literature that LPS induces CD44 expression in monocytes 64,65, however, then why did CD44 cells decrease after *S.* Typhimurium infection in our study? We believe that activation-induced death of splenic cells could contribute to the observed CD44 cell loss during *S.* Typhimurium infection. The role of CD44 expression in

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the development of T_{reg} cells has been reported 66, and therefore, we analyzed $CD44^{high}$ cells of spleens for the presence of $CD4^{+}CD25^{+}$ T_{reg} cells (Figure 5A). We found that $CD44^{high}$ cells contained much higher levels of $CD4^{+}CD25^{+}$ T_{reg} cells than did the $CD44^{med}$ cells in C57BL/6 mice (Figure 5B). Likewise, $CD44^{high}$ cells contained much higher levels of $CD4^{+}CD25^{+}$ T_{reg} cells than did $CD44^{med}$ cells in IL-$10^{-/-}$ mice (Figure 5C).

IL-10 has been shown to be associated with both immune stimulation and suppression of tumor growth. A high incidence of colorectal adenocarcinomas (60%) was observed in IL-$10^{-/-}$ aged mice. The IL-$10^{-/-}$ mouse is a model for colon cancer, which develops via the dysphasia sequence, a process similar to that seen in clinical inflammatory bowel disease-associated cancer 67, 68. Therefore, we next examined IL-$10^{-/-}$ aged mice and found increasingly higher $CD44^{high}$ levels in the splenocytes of aged IL-$10^{-/-}$ mice than young mice (Figure 6B). Moreover, when we injected B16 tumor cells s.c. in IL-$10^{-/-}$ aged mice and selected a few mice having either large or small tumors, we found a higher expression of $CD44^{high}$ in the spleens of mice having larger tumors than in those with smaller tumors (Figure 6C). Aged mice have reportedly expressed higher levels of $CD44^{high}$ than do the younger mice (about two fold) (Figure 6A). For example, $CD44^{high}$ expression was greater in aged SPA-$1^{-/-}$ mice developing late onset of myeloid leukemia 69. Thus, $CD44^{high}$ cells may contribute to tumor occurrence and development. Therefore, the relationship of $CD44^{high}$ expression of lymphocytes with that of tumor growth should be further studied in detail.

To confirm that CD44 is important for tumor growth, we sorted $CD44^{+}$ and $CD44^{-}$ cells from human liver tumor samples. We found $CD44^{+}$ tumor cells grew much faster compared to $CD44^{-}$ tumor cells *in vivo* (Figure 7); however, their growth rates were quite similar *in vitro*. These data suggested to us a dependence on CD44 in tumor development in *vivo*. CD44 is a cell surface proteoglycan, thought to be involved in cell-to-cell adhesion and cell matrix-adhesion interactions, lymphocyte activation, and homing and cell migration. We believe *S.* Typhimurium suppresses tumor growth through various direct and indirect pathways, and that our data provide indication that it directly down-regulated the expression of CD44 in tumors *in vitro* and in lymphocytes *in vivo*. Further, *S.* Typhimurium seemed to enhance the anti-tumor response by reducing the numbers of $CD4^{+}CD25^{+}$ T_{reg} cells.

We noted that the *lppAB/msbB* and *lppB/msbB* mutants induced a very low Th1 response in mice, including minimal interferon (IFN)-γ and TNF-α production and higher levels of IgG1 antibody production, compared to that in the WT *S.* Typhimurium-treated mice which exhibited the opposite trend 17, 70. These data suggested that the Th1-induced cellular responses were more important than the antibody (humoral) responses in anti-tumor immune mechanisms. Would such mutants accordingly be beneficial in controlling tumor growth in humans? The advantage of immunizing first with the mutants is that subsequently higher doses of the WT bacteria can be given without danger of inducing sepsis in the host. Certainly, this adjunct therapy would then seem to be advantageous, as the virulence of the WT *S*. Typhimurium is likely seems to be essential in retarding tumor growth in the host. The *lpp/msbB* mutants in general and the *lppAB/msbB* mutant in particular caused a significantly increase in the down regulation of CD44 and the number of $CD4^{+}CD25^{+}$ T_{reg}

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cells, when compared to the WT *S.* Typhimurium in the splenocytes of infected mice (Figure 2 and Figure 4). These data tend to suggest the role of LPS and Lpp in the down regulation of CD44 and T_{reg} cells, with an ultimate reduction in the tumor suppression function of *lpp/msbB* mutants (Figure 1). An earlier study by Low *et al.* (2) reported that *S*. Typhimurium *msbB* mutant did not alter anti-tumor activity against s.c. implanted B16F10 melanomas 2. Therefore, Lpp might be more important in inducing an anti-tumor effect than LPS and would be studied in the future.

Recently, it was demonstrated that anti-tumor activity of *Salmonella* strains correlated with decreased angiogenesis and increased tissue necrosis within the tumor tissue 71. Their results showed that *Salmonella* could directly inhibit tumor growth. Our data pointed to the fact that *S.* Typhimurium did not only directly inhibit tumor growth but also inhibited tumor growth through indirectly pathways including changes in the host immune system, specifically by down-regulating CD44 and CD4 T_{reg} cell numbers in the spleen of mice.

In this study, we used *S.* Typhimurium mutants *lppAB/msbB* and *lppB/msbB* as adjuncts to help WT *S.* Typhimurium suppress tumor cell growth. We found tumor growth suppression by 88% in mice that were first immunized with the *lppB/msbB* mutant and then challenged with 2×10^3 cfu of WT *S*. Typhimurium. Our data provided possible mechanisms by which *S.* Typhimurium could modulate tumor growth and include down regulation of CD44 and $CD4^+CD25^+$ T_{reg} cell expression, which would, in turn, up-regulate host immune responses to inhibit tumor growth that is largely LPS- and lipoprotein- dependent. The use of mutant *S.* Typhimurium to suppress tumor growth might provide attractive alternatives for the development of immunotherapy for cancer.

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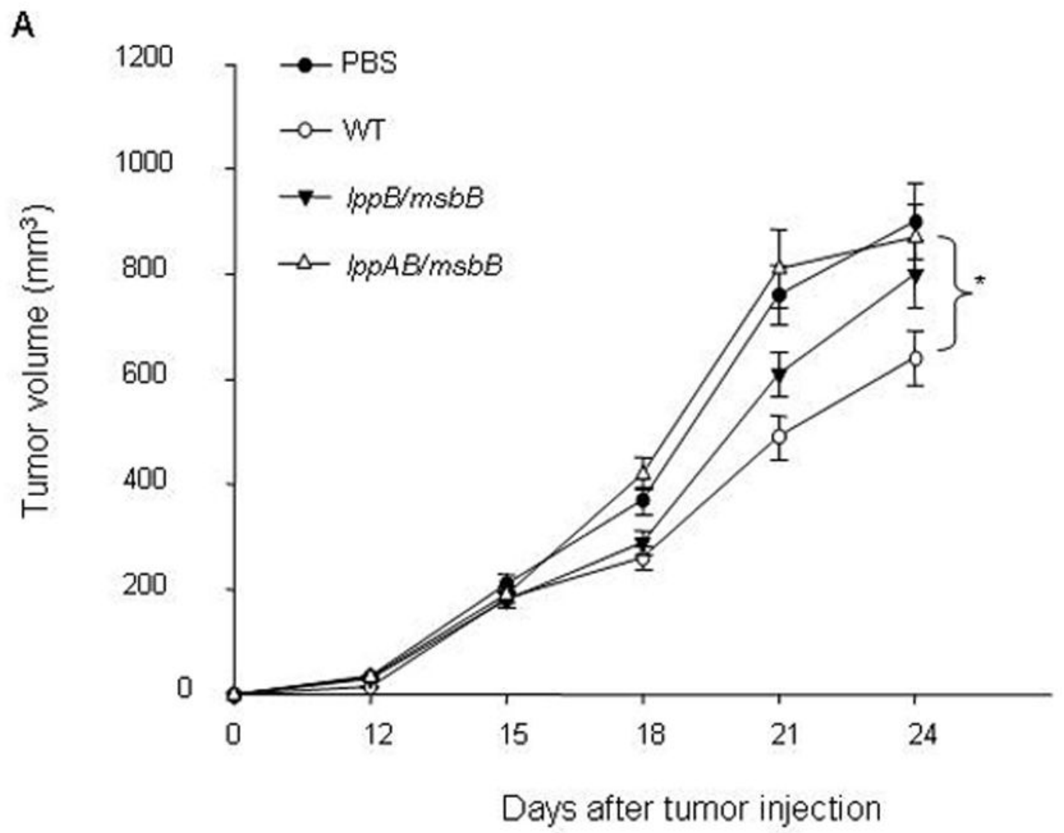

Figure 1A

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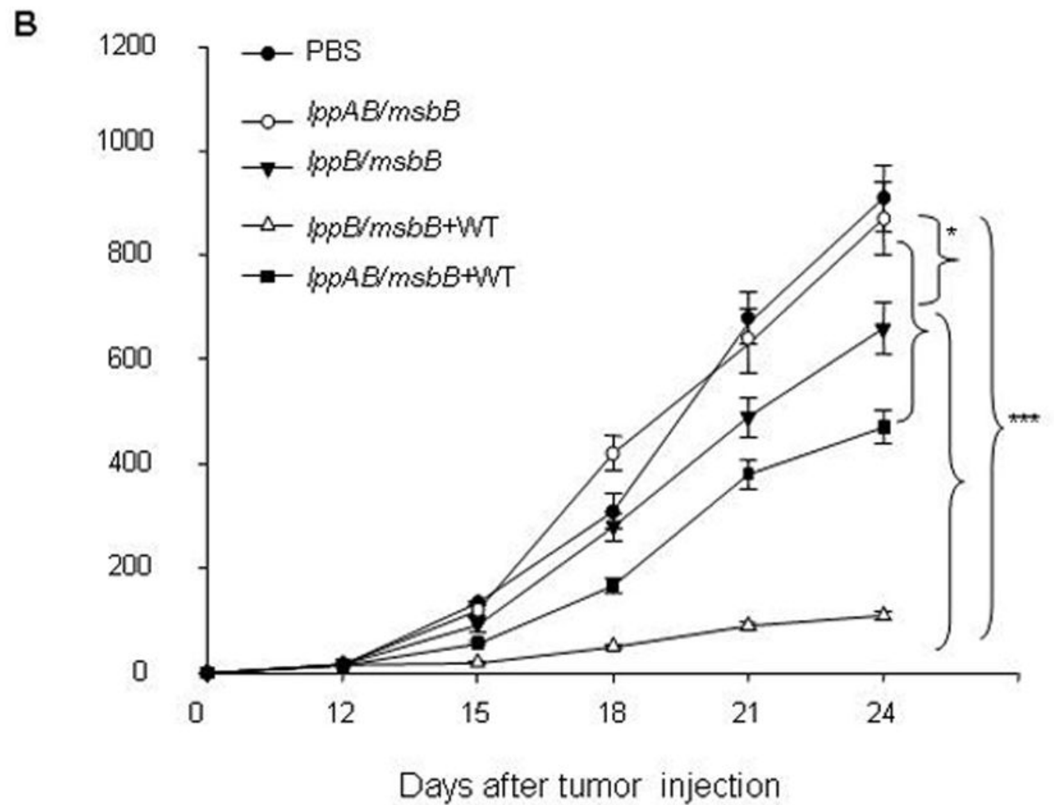

Figure 1B

Figure 1. Suppression of tumor growth in C57BL/6 mice by WT S. Typhimurium and its *lpp/msbB* mutants

A. C57BL/6 mice (n=5/group) were infected via the i.p. route with 50 cfu/100 μl of WT *S*. Typhimurium or its *lppAB/msbB* and *lppB/msbB* mutants, with mice receiving PBS as a control. After 30 days, B16 melanoma cells (5×10^5/100 μl) were subcutaneously injected into mice and the latter observed over a period of 24 days. **B**. Mice were immunized with the *lppAB/msbB* or the *lppB/msbB* mutant at a dose of 2×10^3 cfu. After 30 days, these mice were re-challenged with the WT *S*. Typhimurium at a dose of 2×10^3 cfu. Two weeks later, B16 cells were subcutaneously injected into mice and the sizes of tumors monitored over a period of 24 days. The mean tumor size ± standard deviation is presented. These profiles are representative results from three independent experiments. **S**tatistically significant data are indicated by asterisks (*p<0.05, **p<0.01, and ***p<0.001).

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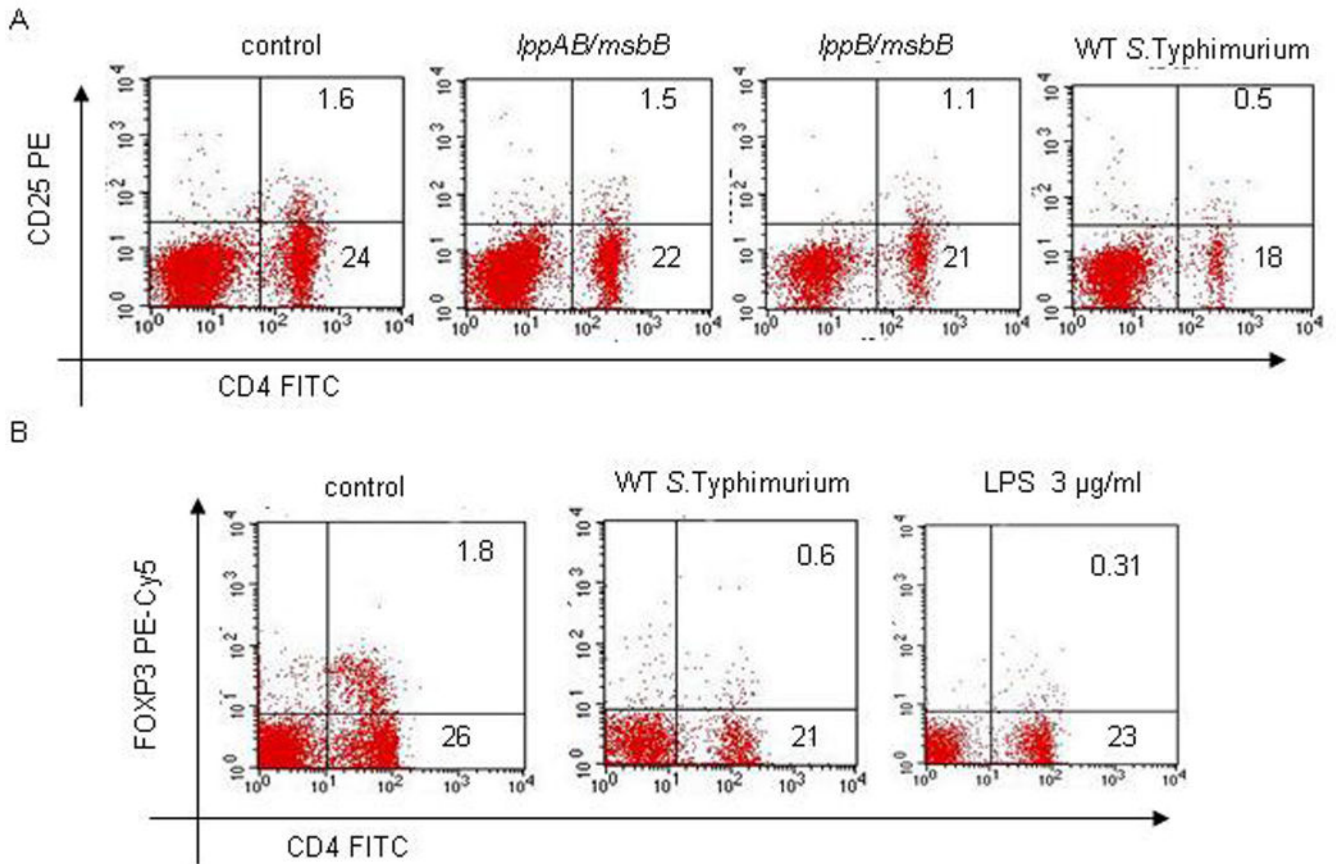

Figure 2.

WT *S.* Typhimurium and its *lppB/msbB* mutant down-regulated $CD4^+CD25^+$ T_{reg} cells in mice. **A**. Swiss-Webster Mice (n=3/group) were infected with WT *S.* Typhimurium or its *lppAB/msbB* and *lppB/msbB* mutant (1 × 103 cfu/mouse). After 5 days, the spleens from infected mice were stained with mouse anti-CD4 FITC and anti-CD25 PE antibodies and splenocytes analyzed by flow cytometry. **B**. Mice splenocytes were cultured with either WT *S*. Typhimurium or LPS (3 μg/ml). After 24 h, the cells were stained with mouse anti-CD4 FITC and Foxp3 PE-Cy5 antibodies and splenocytes analyzed by flow cytometry. These profiles are representative results from three independent experiments.

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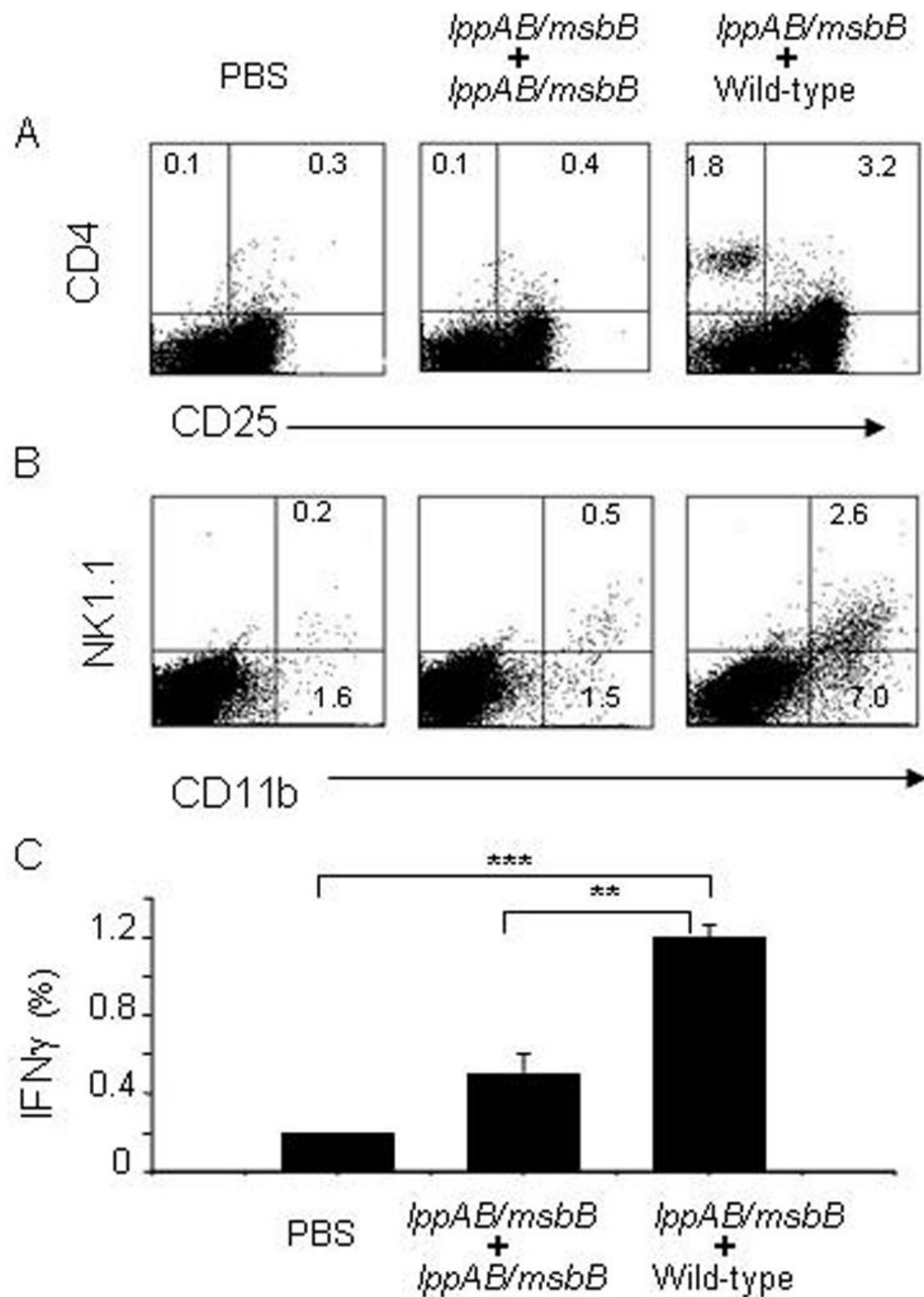

Figure 3.

Infection of immunized mice with WT *S*. Typhimurium increased the number of CD4, $CD4^+CD25^+$, NK cells as well as macrophages in tumors of mice. Mice were immunized with the *lppAB/msbB* mutant at a dose of 2×10^3 cfu. After 30 days, these mice were challenged with the WT *S*. Typhimurium or the *lpp* mutant at a dose of 2×10^3 cfu. Two weeks later, B16 cells were subcutaneously injected into mice, and then three weeks later, tumor cells were removed and stained with CD4-PE-Cy7, CD25-APC, NK1.1-FITC, CD11b-PE, CD3-PE, and IFNγ–FITC antibody for analysis by flow cytometry. These

profiles are representative results from three independent experiments. Statistically significant data are indicated by asterisks (**$p<0.01$,***$p<0.001$).

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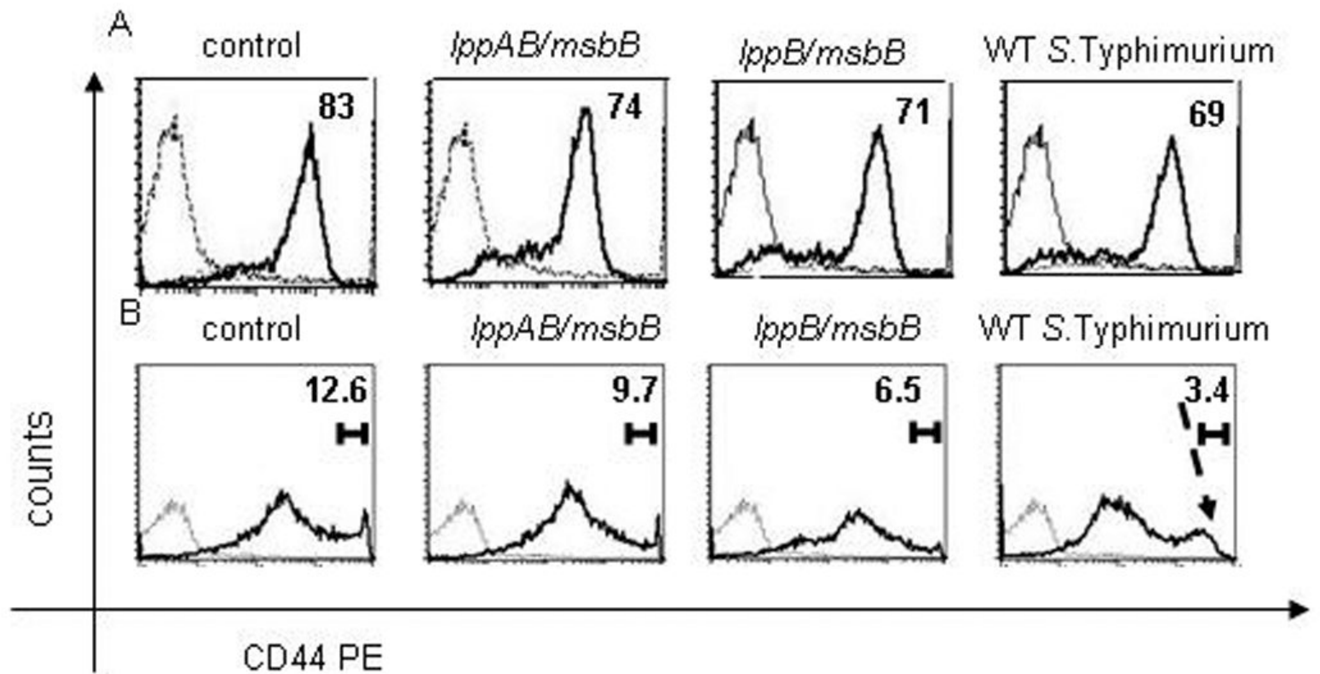

Figure 4.
WT *S.* Typhimurium and its *lpp/msbB* mutant strains down-regulated expression of CD44 in B16 tumor cells and in the splenocytes of mice. **A**. B16 cells were infected with WT *S.* Typhimurium or its *lppB/msbB* and *lppB/msbB* mutants. After 24 h, the cells were stained with mouse anti-CD44 PE. **B**. Swiss-Webster mice (n=3/group) were treated with WT *S.* Typhimurium or its *lppB/msbB* and *lppB/msbB* mutants, with uninfected mice as negative controls. After 5 days, the splenocytes from infected and uninfected mice were stained with mouse anti-CD4 PE antibody and the data analyzed by flow cytometry. An isotype control is shown as a light-colored line on the left of each panel. These profiles are representative results from three independent experiments.

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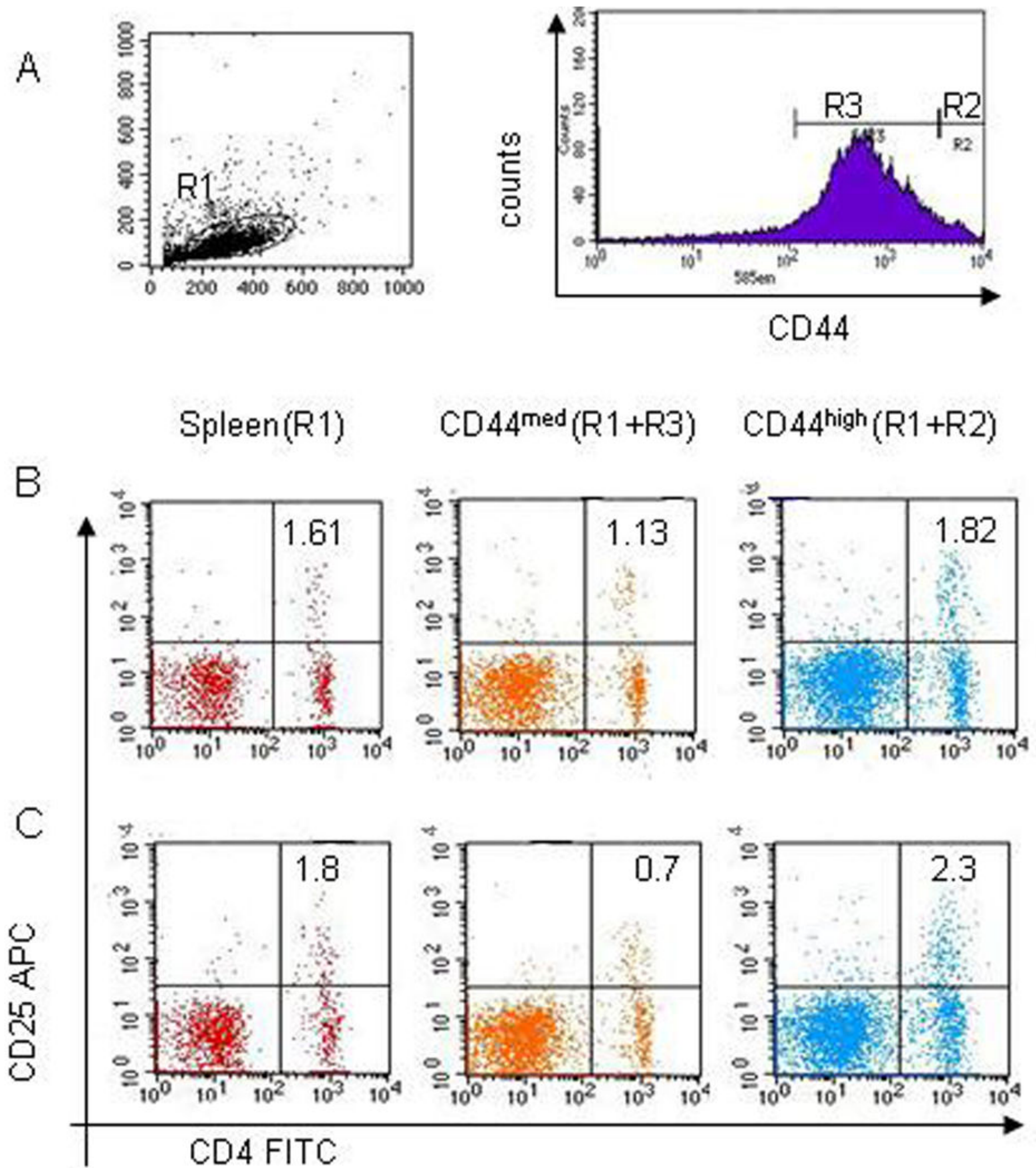

Figure 5.

The population of $CD4^+CD25^+$ T_{reg} cells in $CD44^{high}$ cells from spleens of C57BL/6 and $IL\text{-}10^{-/-}$ aged mice. Splenocytes from C57BL/6 and $IL\text{-}10^{-/-}$ aged mice (3=/group) were stained with mouse anti-CD44 PE, anti-CD4 FITC and anti-CD25 APC antibodies, and analyzed by flow cytometry. **A**. $CD44^{med}$ and $CD44^{high}$ cells were gated. In splenocytes, R1= lymphocytes, R1+R2= $CD44^{high}$ cells R1+R3=$CD44^{med}$. **B**. $CD4^+CD25^+$CD44 cells in C57BL/6 mice. **C**. $CD4^+CD25^+$ CD44 cells in $IL\text{-}10^{-/-}$ aged mice. These profiles are representative results from three independent experiments.

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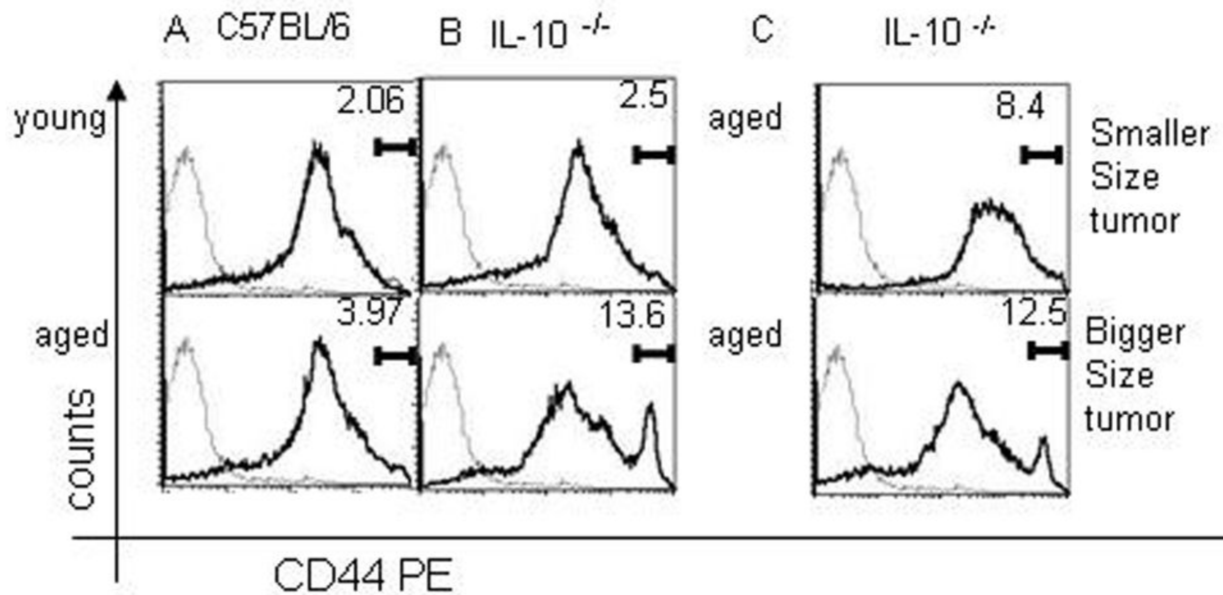

Figure 6.

IL-10$^{-/-}$ aged mice (10/group) were subcutaneously injected with B16 tumor cells. After 24 days, the mice with bigger or smaller tumors were sacrificed, and the splenocytes were stained with mouse anti-CD44 PE antibodies. These stained cells were then analyzed with flow cytometry. **A**. CD44 expression in the spleens of C57BL/6 young and aged mice. **B**. CD44 expression in IL-10 $^{-/-}$ young and aged mice. **C**. CD44 expression in the smaller and larger tumors of IL-10$^{-/-}$ aged mice. An isotype control is shown as a light-colored line on the left of each panel. These profiles are representative results from three independent experiments.

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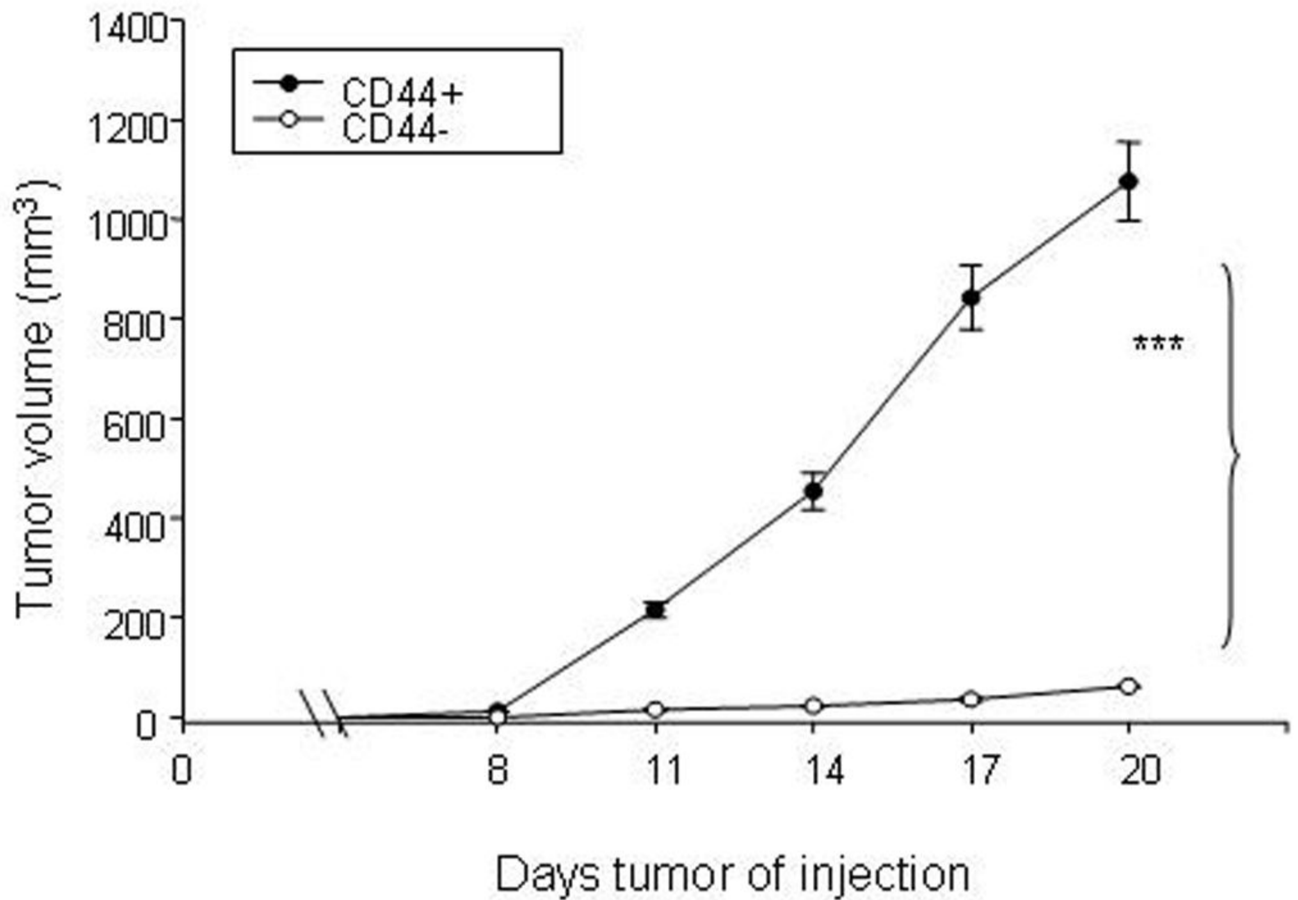

Figure 7.

Growth of human CD44 liver cancer cells in mice. Both $CD44^+$ and $CD44^-$ cells from human liver cancer were sorted and subcutaneously injected into BALB/c mice (n=5/group). The tumor size was measured over a period of 20 days. The mean tumor size ± standard deviation is presented. These profiles are representative results from three independent experiments. Statistically significant data are indicated by asterisks (*** $p<0.001$).

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TABLE 1

Strains used in this study

Strain	Relevant characteristic(s)	Reference
S. enterica serovar Typhimurium strains		
14028	*S. enterica* serovar Typhimurium	72
Mutant *lppB*/*msbB*	Isogenic mutant in which the *lppB* gene was deleted from the *msbB* minus background strain of 14028; Nal^r Tc^r Kn^r	70
Mutant *lppAB*/*msbB*	Isogonics mutant in which the *lppAB* genes were deleted from the *msbB* minus background strain of 14028; Nal^r Tc^r Kn^r	73

Nal^r = Nalidixic acid resistance; Tc^r = Tetracycline resistance; Kn^r = Kanamycin resistance