Attenuated *Salmonella enterica* Typhimurium Reduces Tumor Burden in an Autochthonous Breast Cancer Model

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Abstract. *Background/Aim: Cancer treatment with attenuated Salmonella enterica Typhimurium (S. Typhimurium) has gained momentum in recent years. However, the effectiveness of this treatment has not been explored in autochthonous models. We report the efficacy of S. Typhimurium in mice with autochthonous mammary tumors. Materials and Methods: S. Typhimurium attenuated by deletion of cyclic adenosine monophosphate signaling, SalpNG.1, was injected into female BALB-neuT tumor-bearing mice. Mice were monitored for efficacy and sacrificed for mechanistic studies. Results: In treated mice, seven-week post-treatment tumor burden was reduced by 85% and median survival was increased by 88%. Efficacy was correlated with increased tumor-infiltrating CD8 and natural killer cells. In addition, SalpNG.1 treatment caused a systemic increase of monocytic myeloid-derived suppressor cells that accumulated to high numbers within tumor tissue. Bacteria were not detected in tumor tissue, suggesting that the observed efficacy was due to a systemic rather than a tumor-specific effect of the bacteria. Conclusion: S. Typhimurium treatment reduces tumor burden and increases survival in an autochthonous breast cancer model.*

The National Cancer Institute Surveillance, Epidemiology and End Results Program estimates that 1 in 2 men and 1 in 3 women will develop cancer in their lifetime. In the United States alone, nearly every minute of every day a person dies due to cancer resulting in approximately 500,000 cancer deaths each year. These statistics underpin the urgent need for more effective cancer therapies.

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Through the process of cancer immunoediting, nascent tumors develop immune hallmarks that are required for growth and progression. These include the ability to thrive in a chronically inflamed microenvironment, the ability to evade immune recognition and the ability to suppress immune reactivity (1). Several therapies have been or are currently under development to reverse the immunosuppressive hallmarks and promote immune-mediated tumor clearance. These include, but are not limited to, administration of immune-activating cytokines, antibody blockade of immunosuppressive proteins and immunoinhibitory checkpoints, adoptive transfer of cytotoxic cells and several cancer vaccination strategies (2-4). While met with varying levels of success, these strategies are often accompanied by limiting toxicities that are the result of an accelerated immune system (5, 6).

Long before the advent of anticancer immunotherapy, William Coley injected cancer patients with a combination of heat-killed *Streptococcus pyogenes* and *Serratia marcescens* (Coley's toxins) (7). The use of Coley's toxins was eventually discontinued due to the growing popularity of surgery, radiation and chemotherapy. Recently, in light of increased understanding of the interaction between microorganisms and the mammalian immune system, there is renewed interest in bacterial cancer therapy (8, 9). Bacteria, particularly Gram-negative facultative anaerobes, such as *Salmonella*, have several characteristics that make them prime candidates for cancer therapy (8). *S. Typhimurium* has demonstrated the propensity to specifically accumulate within the tumor microenvironment with ratios greater than 1,000:1 compared to tissues normally infected by the bacteria (10). In addition, the genetic manipulability of *S. Typhimurium* allows for the expression of foreign recombinant proteins, making these bacteria an effective delivery system for proteins that may be toxic when administered systemically. Moreover, *S. Typhimurium* has displayed natural anti-tumor efficacy by directly killing tumor cells through pyroptosis and indirectly stimulating an immune response in the tumor microenvironment leading to the activation of cytotoxic CD8 and natural killer (NK) cells (8).

There have been several reports of pre-clinical efficacy using virulence-attenuated strains of *S. Typhimurium*, including but not limited to: A1-R, which is auxotrophic for leucine and arginine (11), SL7207 and related strains, which are auxotrophic for aromatic amino acids (12-14) and perhaps the most widely used strain VNP20009, which lacks lipopolysaccharide (LPS) and is auxotrophic for purines (15). The anticancer efficacy of VNP20009 has been demonstrated in several pre-clinical studies (16-22); however, when tested clinically, very few tumor biopsies of treated patients contained colony forming units (cfu) of the bacteria, indicating a problem with tumor targeting in clinical disease (23-25). Lack of clinical efficacy has since been attributed by some to an over-attenuation of the strain due to its LPS deficiency, which may be important in eliciting a tumor necrosis factor response that opens vasculature and allows a blood influx to carry the bacteria into the tumor (9).

Several potential cancer immunotherapies have demonstrated promising preclinical results, yet have yielded disappointing clinical results. This may be attributed in part to the artificiality of many preclinical animal models. Transplantation models of cancer do not adequately mimic the complex interplay between developing tumors and the host immune system, and therefore, treatments based on efficacy observed in these models often yield disappointing clinical results. As a more realistic alternative, genetically-engineered mouse models (GEMM) have been generated that more slowly develop autochthonous tumors from a single cell and, therefore, more closely mimic human disease (26). The BALB-neuT model is a GEMM of invasive breast cancer on the BALB/c background that expresses a constitutively active rat Her2 receptor (neu) driven by the mouse mammary tumor virus promoter. In this model, cancer develops over several months and appears in the mammary pads of female mice as palpable tumors around 16 weeks of age. The tumors closely resemble the aggressive Her2-driven cancer found in human patients (27).

We are developing a bacterial anticancer therapy based on *S. Typhimurium* strain χ4550, which is attenuated by knockout of cyclic adenosine monophosphate signaling (28). This strain has demonstrated tumor-targeting propensity and antitumor efficacy in several transplantation mouse models of cancer (29-32). Herein, we report the efficacy of strain χ4550 in the BALB-neuT breast cancer GEMM illustrating its potential for the treatment of autochthonous tumors.

Materials and Methods

Bacterial strain and maintenance. The attenuated *S. Typhimurium* strain χ4550 (Δcya, Δcrp, Δasd) (28) was a gift from Dr. Roy Curtiss III (Arizona State University). SalpNG.1 was constructed by transforming χ4550 with pNG.1, a plasmid containing cDNA coding for aspartate semialdehyde dehydrogenase to complement the χ4550 requirement for diaminopimelic acid. To construct plasmid pNG.1, plasmid pYA292 (33) was cut with *EcoRI* and *HindIII*, the ends filled in, and the plasmid recircularized to eliminate the *LacZ(alpha)* coding sequence. Overnight cultures of SalpNG.1 were grown in lysogeny broth (LB) and flash frozen with liquid nitrogen in 15% glycerol in LB and stored at –80˚C. Before treatment, bacteria were thawed at 37˚C and appropriately diluted in phosphate-buffered saline (PBS).

BALB-neut tumor studies. BALB-neuT mice were maintained in specific pathogen-free conditions and fed standard mouse chow. Animals were cared for by the University of Minnesota's Research Animal Resources, and all animal use was approved by the University's Institutional Animal Care and Use Facility. Genotyping for the neu transgene was performed by Transnetyx on male and female pups (Memphis, TN, USA). Breeding pairs consisted of heterozygous males and homozygous negative females. Female mice that were positive for the *neu* transgene were monitored for tumor development. Upon initial tumor palpation (~16 weeks), mice were injected intravenously with 6×10^4 cfu of SalpNG.1. Tumors were measured twice weekly with a digital caliper, and individual tumor volumes were calculated as spheroid ($L\times W^2\times0.52$). Individual tumor volumes were added to give total tumor burden for each mouse. For survival studies, mice were monitored for survival and euthanized if they became moribund or if a single tumor reached 2 cm^3 in volume.

Bacterial colonization experiments. For the bacterial colonization assays, a set of tumor-bearing mice was sacrificed 21 days post-treatment. Spleens and tumors were harvested from mice and minced to ~1 mm^3 pieces. Resulting samples were homogenized in 5 ml of sterile PBS in gentleMACS M Tubes with Strainers using a gentleMACS Tissue Dissociator and running program RNA_01 (Miltenyi Biotec, Bergich Gladbach, Germany). The homogenate was serially diluted in sterile PBS and plated on LB agar containing 100 μg/ml nalidixic acid for selection. Plates were incubated overnight at 37˚C, and cfu were enumerated the next day.

Flow cytometry. BALB-neuT mice were sacrificed 21 days after treatment. Tumors and spleens were homogenized using a mouse Tumor Dissociation Kit (Miltenyi Biotec) according to manufacturer's instructions. Cell suspensions were stained and analyzed on an LSRII flow cytometer (BD Biosciences, San Jose, CA, USA). Cells were gated and identified as follows: CD8 T-cells ($CD8^+$), NK cells ($CD8^-$, $CD4^-$, $CD49b^+$), monocytic myeloid-derived suppressor cells (MDSCs) ($CD45^+$, $CD11b^+$, $Ly6C^{Hi}$), granulocytic MDSCs ($CD45^+$, $CD11b^+$, $Ly6G^{Hi}$). Cells/sample were enumerated using CountBright absolute counting beads (Life Technologies, Grand Island, NY, USA). Cells/gram measurements were calculated based on the percent of sample analyzed, tumor weight and homogenate volume. Antibody conjugates were purchased from BioLegend (San Diego, CA, USA): αCD8/FITC (100705), αCD4/PerCP-Cy5.5 (100539), αCD49b/PE/Cy7 (108921), αCD45.2/BV510 (109837), αCD11b/BV650 (101239) and BD Pharmingen (San Jose, CA, USA): αLy6C/PerCP/Cy5.5 (560525) and αLy6G/AF700 (561236).

Results

Treatment with S. Typhimurium reduces tumor burden in BALB-neuT mice. Mammary fat pad tumors in female BALB-neuT mice became palpable (~50-60 mm^3) around 16

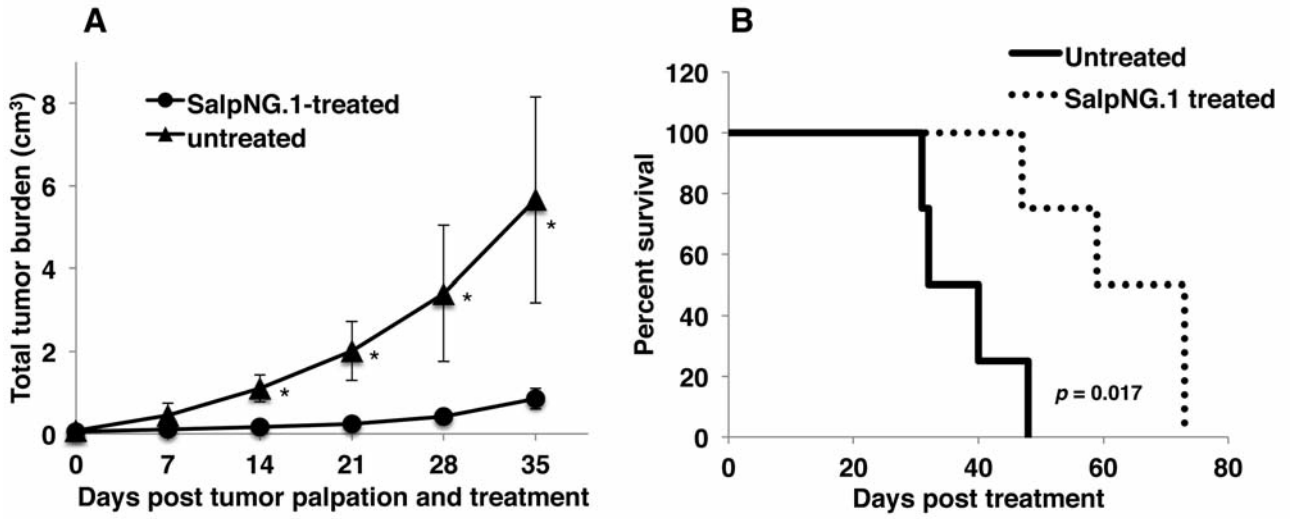

Figure 1. *SalpNG.1 reduces tumor burden and increases survival of BALB-neuT mice with autochthonous tumors. A: Mice were monitored for tumor development. Upon tumor palpation (day 0), mice were either treated with a single dose of 6×10^4 cfu of SalpNG.1 or left untreated, and total tumor size was measured over time. Data displayed is an average (±standard deviation) of 4 mice per group. *=p-value <0.05 B: Kaplan-Meier survival plot of treated and untreated mice shown in panel A (p-value was generated using the log-rank test).*

weeks of age. At this time (day 0), the mice typically had 1-3 palpable tumors. These tumors enlarged over time, and new tumors appeared on the remaining fat pads, usually until each mammary pad developed a tumor. Individual tumors were measured weekly by caliper, and their volume was calculated and combined to give a total tumor burden measurement for each mouse. When left untreated, the average total tumor burden per mouse reached 5.66 cm^3 by day 35 (Figure 1A). In contrast, if the mice were given a single intravenous injection of SalpNG.1 at day 0, tumor progression was significantly delayed, and average total tumor burden at day 35 was only 0.85 cm^3, corresponding to an 85% tumor reduction (Figure 1A). This correlated to a significant increase in survival time of SalpNG.1-treated mice (median, 66 days) when compared to untreated mice (median, 35 days) (Figure 1B). In order to test if multiple injections of SalpNG.1 improved efficacy, the experiments were repeated with mice treated on day 0 and every 21 days following. No difference in tumor burden or survival was observed between single and multiple treatments.

The propensity of *S. Typhimurium* to preferentially accumulate in tumor tissue over healthy tissues has been extensively reported (8). However, these observations have been from experiments performed in subcutaneous and orthotopic transplant models in mice. We sought to determine the tumor-targeting propensity of SalpNG.1 in autochthonous tumors in the BALB-neuT model. Twenty-one days following treatment with SalpNG.1, tumors and spleens were removed, homogenized and plated onto selective nutrient agar for colony formation. No cfu were found in any of the processed tumors. In contrast, cfu were found in each spleen tested, with an average of $1.5\times10^4\pm9.5\times10^3$ cfu/gram tissue.

Efficacy of S. Typhimurium is correlated with increased tumor-infiltrating lymphocytes. An important role of NK cells and CD8 T-cells in the anticancer efficacy of χ4550-based strains has been demonstrated using transplant models (32). In order to examine the role of these immune cells in BALB-neuT breast cancer, tumors from mice were homogenized on day 21 post-treatment, and tumor-infiltrating lymphocytes were isolated for characterization using flow cytometry. Tumors from treated mice contained greater than 10-fold more infiltrating CD8 T-cells than tumors from untreated mice. Similarly, infiltrating NK cell numbers were approximately 6-fold greater in treated mice indicating an immune response in reaction to SalpNG.1 treatment (Figure 2). CD4 T-cells were also examined, but the difference in numbers between groups was insignificant.

Treatment with S. Typhimurium increases monocytic MDSCs both systemically and within tumors. In spite of a reduction in tumor burden and an increase in survival time, eventually all treated mice succumbed to disease. We examined changes in MDSC numbers as a potential contributor to tumor progression. MDSCs are a suppressive subset of immature myeloid cells that express CD11b and the Gr-1 epitope and are present in several cancer models including BALB-neuT breast cancer (27). MDSCs are conventionally

subdivided into monocytic ($Ly6C^{Hi}$) and granulocytic ($Ly6G^{Hi}$) subsets and, while both subsets have been implicated in tumor immunosuppression and cancer progression, monocytic MDSCs have been shown to be more consistently immunosuppressive on a per-cell basis than granulocytic MDSCs (34). We investigated whether SalpNG.1 treatment affected MDSC presence in tumor tissue. When examined 21 days post-treatment, tumors from treated mice displayed a greater than 10-fold increase in monocytic MDSCs, while granulocytic MDSCs were not significantly affected (Figure 3A-B). In addition, the ratio of monocytic to granulocytic MDSCs in the spleen was increased in response to treatment, suggesting that the increase in tumor-infiltrating monocytic MDSCs was due to a systemic immunological effect of the bacteria rather than a tumor-specific effect (Figure 3C).

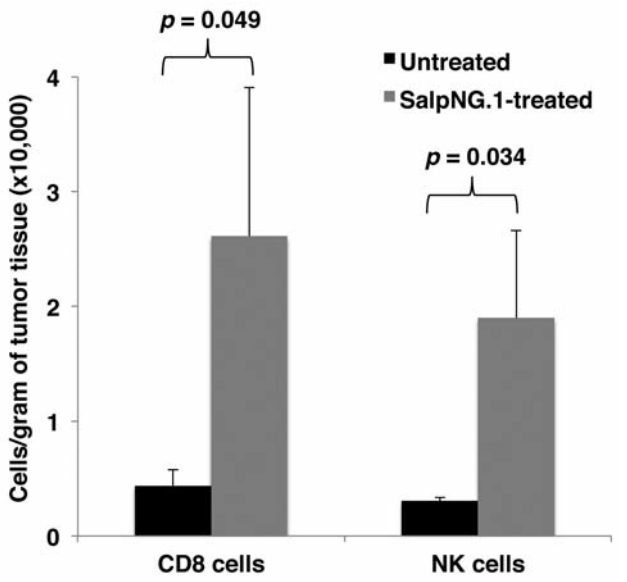

Figure 2. *SalpNG.1 treatment increases the quantity of tumor-infiltrating cytotoxic lymphocytes. Infiltrating lymphocytes were isolated from the tumors of treated or untreated mice (3 mice each). Cells were labeled with antibodies and analyzed using flow cytometry. CD8 and NK cells per gram were calculated based on tumor mass and cells counted. Error bars indicate standard deviation, and p-values are based on the Student's unpaired t-test.*

Discussion

Treatment of tumor-burdened BALB-neuT mice with *S. Typhimurium* resulted in delayed tumor growth and a survival advantage. While the anticancer efficacy of *S. Typhimurium* has been demonstrated in several transplant models (8), the results reported here demonstrate *S. Typhimurium*'s efficacy in an autochthonous tumor model. The reduction in tumor burden was accompanied by increased infiltration of cytotoxic T-cells and NK cells, which are important subsets of immune cells in cancer immunotherapy.

The presence of tumor-infiltrating CD8 T-cells is associated with a positive prognosis in the majority of human cancers (35). In addition, T-helper-1 (Th1) polarization is important for an effective anti-tumor immune response (35, 36). *S. Typhimurium* is a strong elicitor of the Th1 response, and treatment with SalpNG.1 may have caused a systemic Th1 shift resulting in the infiltration of cytotoxic lymphocytes leading to the anti-tumor efficacy observed.

Interestingly, in spite of the efficacy, we found no bacteria within the tumor tissue; therefore, we conclude that the observed efficacy and immune cell recruitment were due to a systemic immune stimulation rather than a tumor microenvironment-specific stimulation. The lack of tumor colonization in the BALB-neuT model was unexpected given the empirical evidence of attenuated *S. Typhimurium* strains accumulating to high levels in tumors in other models. This observation may be attributed to differences in tumor development and microenvironment physiology between autochthonous tumors and tumors developed in transplantation models (26). At the time bacteria were administered to the mice in our experiments, the tumors may not have been large enough to sufficiently develop the hypoxic and necrotic areas shown to be important for tumor colonization (13, 37). Future experiments will investigate methods to improve tumor targeting in this model, including the treatment of larger, more established tumors. Also, altering the injection strategy to include a "primer" injection, which was shown to increase tumor targeting and efficacy in a murine Lewis lung cancer model, may potentiate tumor targeting in the BALB-neuT model (38).

It is becoming increasingly clear that necrosis in tumors is important for bacterial colonization (37). Early necrosis in transplanted tumor models is often caused by the death of a majority of the inoculated tumor cells leading to an early inflammatory response and a necrotic center (39, 40). Since this necrosis is absent in early-stage autochthonous tumors, future studies might benefit from the inclusion of radiation therapy or other strategies to cause necrosis of tumor tissue and allow colonization to the degree that is observed in transplant models. Also, differences in vasculature development between autochthonous and transplant tumor models have lead to varying responses to treatments, such as interleukin-12 (41). These differences may have similar implications in bacterial tumor colonization. The more chaotic and less mature vasculature that characterizes transplantation tumors may more readily hemorrhage in response to *S. Typhimurium* than the more mature vasculature of autochthonous tumors. This may allow the bacteria to more easily colonize transplantation tumors than

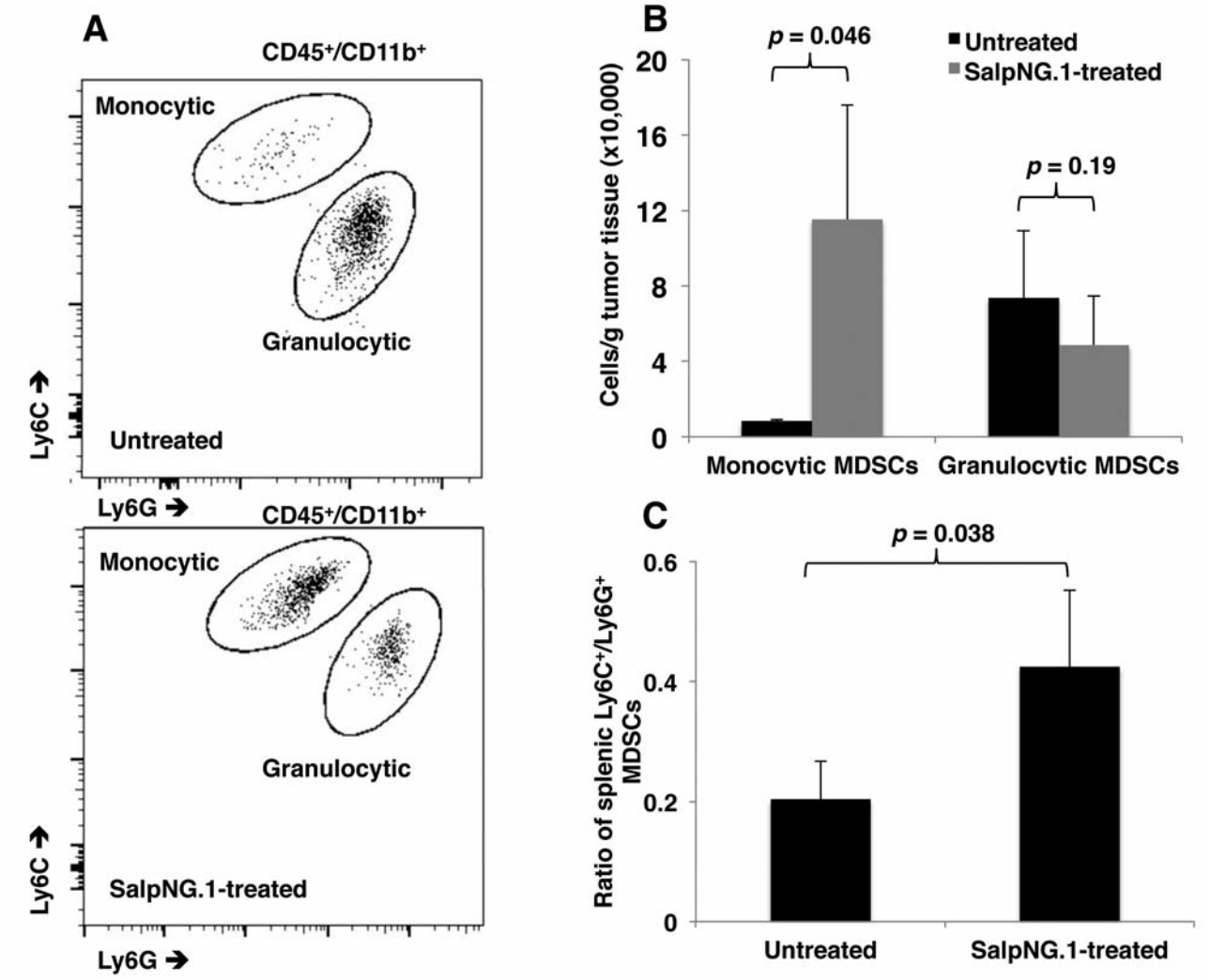

Figure 3. *SalpNG.1 treatment causes enrichment of monocytic MDSCs. MDSCs in tumor and spleen homogenates from treated or untreated mice were labeled with antibodies and identified using flow cytometry as monocytic (Ly6C^{Hi}) or granulocytic (Ly6G^{Hi}). A: Representative plots from tumor samples. B: Resulting cells per gram of tumor tissue were quantified for multiple mice. C: The average ratio of monocytic/granulocytic MDSCs in spleens from treated or untreated mice (3 mice each). The student's unpaired t-test was used to calculate p-values.*

autochthonous tumors as hemorrhaging of tumor blood vessels is likely required for infiltration of the bacteria into the tumor tissue (13). The differences in tumor targeting between preclinical transplant and autochthonous models could potentially explain the lack of bacterial colonization in tumor biopsies of reported clinical trials (23-25) using attenuated *S. Typhimurium*. Achieving tumor targeting in an autochthonous model will likely be more predictive of successful human tumor targeting.

Along with cytotoxic lymphocytes, we observed an increased number of monocytic MDSCs in the tumor tissue of SalpNG.1-treated mice. This change was likely due to systemic immune-modulatory effects of the bacteria rather than a tumor-specific response, as we also observed an enrichment of monocytic MDSCs in the spleen. The LPS surface molecules on *S. Typhimurium* have been shown to increase the presence and suppressive activity of MDSCs (42, 43), and a recent report demonstrated the recruitment of monocytic MDSCs from the bone marrow to tissues during *S. Typhimurium* infection (44). The potential efficacy of *S. Typhimurim*-based cancer therapy may not be fully realized until MDSC recruitment and/or inhibitory activity is addressed. Current experiments are underway to investigate inhibitors of MDSC function that could be used in combination with SalpNG.1 treatment to improve efficacy.

Conflicts of Interest

All Authors declare no conflict of interest and disclose no financial interest in the contents of this manuscript.

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References

1 Cavallo F, De Giovanni C, Nanni P, Forni G and Lollini PL: 2011: the immune hallmarks of cancer. Cancer immunology, immunotherapy: CII *60*: 319-326, 2011.

2 Aranda F, Vacchelli E, Eggermont A, Galon J, Sautes-Fridman C, Tartour E, Zitvogel L, Kroemer G and Galluzzi L: Trial Watch: Peptide vaccines in cancer therapy. Oncoimmunology *2*: e26621, 2013.

3 Pardoll DM: The blockade of immune checkpoints in cancer immunotherapy. Nat Rev Cancer *12*: 252-264, 2012.

4 Vacchelli E, Aranda F, Obrist F, Eggermont A, Galon J, Cremer I, Zitvogel L, Kroemer G and Galluzzi L: Trial watch: Immunostimulatory cytokines in cancer therapy. Oncoimmunology *3*: e29030, 2014.

5 Alatrash G, Jakher H, Stafford PD and Mittendorf EA: Cancer immunotherapies, their safety and toxicity. Expert Opin Drug Saf *12*: 631-645, 2013.

6 Schwartz RN, Stover L and Dutcher J: Managing toxicities of high-dose interleukin-2. Oncology *16*: 11-20, 2002.

7 McCarthy EF: The toxins of William B. Coley and the treatment of bone and soft-tissue sarcomas. The Iowa orthopaedic journal *26*: 154-158, 2006.

8 Forbes NS: Engineering the perfect (bacterial) cancer therapy. Nat Rev Cancer *10*: 785-794, 2010.

9 Leschner S and Weiss S: *Salmonella*-allies in the fight against cancer. J Mol Med *88*: 763-773, 2010.

10 Pawelek JM, Low KB and Bermudes D: Tumor-targeted *Salmonella* as a novel anticancer vector. Cancer Res *57*: 4537-4544, 1997.

11 Hoffman RM and Zhao M: Methods for the development of tumor-targeting bacteria. Expert opinion on drug discovery *9*: 741-750, 2014.

12 Yu YA, Shabahang S, Timiryasova TM, Zhang Q, Beltz R, Gentschev I, Goebel W and Szalay AA: Visualization of tumors and metastases in live animals with bacteria and vaccinia virus encoding light-emitting proteins. Nat Biotechnol *22*: 313-320, 2004.

13 Leschner S, Westphal K, Dietrich N, Viegas N, Jablonska J, Lyszkiewicz M, Lienenklaus S, Falk W, Gekara N, Loessner H and Weiss S: Tumor invasion of *Salmonella* enterica serovar Typhimurium is accompanied by strong hemorrhage promoted by TNF-alpha. PLoS ONE *4*: e6692, 2009.

14 Westphal K, Leschner S, Jablonska J, Loessner H and Weiss S: Containment of tumor-colonizing bacteria by host neutrophils. Cancer Res *68*: 2952-2960, 2008.

15 Low KB, Ittensohn M, Le T, Platt J, Sodi S, Amoss M, Ash O, Carmichael E, Chakraborty A, Fischer J, Lin SL, Luo X, Miller SI, Zheng L, King I, Pawelek JM and Bermudes D: Lipid A mutant *Salmonella* with suppressed virulence and TNFalpha induction retain tumor-targeting in vivo. Nat Biotechnol *17*: 37-41, 1999.

16 Luo X, Li Z, Lin S, Le T, Ittensohn M, Bermudes D, Runyab JD, Shen SY, Chen J, King IC and Zheng LM: Antitumor effect of VNP20009, an attenuated *Salmonella*, in murine tumor models. Oncol Res *12*: 501-508, 2001.

17 Loeffler M, Le'Negrate G, Krajewska M and Reed JC: Inhibition of tumor growth using salmonella expressing Fas ligand. J Natl Cancer Inst *100*: 1113-1116, 2008.

18 Loeffler M, Le'Negrate G, Krajewska M and Reed JC: IL-18-producing *Salmonella* inhibit tumor growth. Cancer Gene Ther *15*: 787-794, 2008.

19 Loeffler M, Le'Negrate G, Krajewska M and Reed JC: *Salmonella typhimurium* engineered to produce CCL21 inhibit tumor growth. Cancer immunology, immunotherapy: CII *58*: 769-775, 2009.

20 Loeffler M, Le'Negrate G, Krajewska M and Reed JC: Attenuated *Salmonella* engineered to produce human cytokine LIGHT inhibit tumor growth. Proc Natl Acad Sci USA *104*: 12879-12883, 2007.

21 Lancaster LE, Wintermeyer W and Rodnina MV: Colicins and their potential in cancer treatment. Blood cells, molecules & diseases *38*: 15-18, 2007.

22 Blache CA, Manuel ER, Kaltcheva TI, Wong AN, Ellenhorn JD, Blazar BR and Diamond DJ: Systemic delivery of *Salmonella typhimurium* transformed with IDO shRNA enhances intratumoral vector colonization and suppresses tumor growth. Cancer Res *72*: 6447-6456, 2012.

23 Heimann DM and Rosenberg SA: Continuous intravenous administration of live genetically modified salmonella typhimurium in patients with metastatic melanoma. J Immunother *26*: 179-180, 2003.

24 Toso JF, Gill VJ, Hwu P, Marincola FM, Restifo NP, Schwartzentruber DJ, Sherry RM, Topalian SL, Yang JC, Stock F, Freezer LJ, Morton KE, Seipp C, Haworth L, Mavroukakis S, White D, MacDonald S, Mao J, Sznol M and Rosenberg SA: Phase I study of the intravenous administration of attenuated *Salmonella typhimurium* to patients with metastatic melanoma. Journal of clinical oncology: official journal of the American Society of Clinical Oncology *20*: 142-152, 2002.

25 Nemunaitis J, Cunningham C, Senzer N, Kuhn J, Cramm J, Litz C, Cavagnolo R, Cahill A, Clairmont C and Sznol M: Pilot trial of genetically modified, attenuated *Salmonella* expressing the E. coli cytosine deaminase gene in refractory cancer patients. Cancer Gene Ther *10*: 737-744, 2003.

26 Langdon SP: Animal modeling of cancer pathology and studying tumor response to therapy. Curr Drug Targets *13*: 1535-1547, 2012.

27 Conti L, Ruiu R, Barutello G, Macagno M, Bandini S, Cavallo F and Lanzardo S: Microenvironment, Oncoantigens, and Antitumor Vaccination: Lessons Learned from BALB-neuT Mice. BioMed research international *2014*: 534969, 2014.

28 Curtiss R, 3rd and Kelly SM: *Salmonella typhimurium* deletion mutants lacking adenylate cyclase and cyclic AMP receptor protein are avirulent and immunogenic. Infect Immun *55*: 3035-3043, 1987.
29 Soto LJ, 3rd, Sorenson BS, Kim AS, Feltis BA, Leonard AS and Saltzman DA: Attenuated *Salmonella typhimurium* prevents the establishment of unresectable hepatic metastases and improves survival in a murine model. J Pediatr Surg *38*: 1075-1079, 2003.
30 Barnett SJ, Soto LJ, 3rd, Sorenson BS, Nelson BW, Leonard AS and Saltzman DA: Attenuated *Salmonella typhimurium* invades and decreases tumor burden in neuroblastoma. J Pediatr Surg *40*: 993-997; discussion 997-998, 2005.
31 Saltzman DA, Heise CP, Hasz DE, Zebede M, Kelly SM, Curtiss R, 3rd, Leonard AS and Anderson PM: Attenuated *Salmonella typhimurium* containing interleukin-2 decreases MC-38 hepatic metastases: a novel anti-tumor agent. Cancer Biother Radiopharm *11*: 145-153, 1996.
32 Saltzman DA, Katsanis E, Heise CP, Hasz DE, Vigdorovich V, Kelly SM, Curtiss R, 3rd, Leonard AS and Anderson PM: Antitumor mechanisms of attenuated *Salmonella typhimurium* containing the gene for human interleukin-2: a novel antitumor agent? J Pediatr Surg *32*: 301-306, 1997.
33 Galan JE, Nakayama K and Curtiss R, 3rd: Cloning and characterization of the asd gene of *Salmonella typhimurium*: use in stable maintenance of recombinant plasmids in *Salmonella* vaccine strains. Gene *94*: 29-35, 1990.
34 Dolcetti L, Peranzoni E, Ugel S, Marigo I, Fernandez Gomez A, Mesa C, Geilich M, Winkels G, Traggiai E, Casati A, Grassi F and Bronte V: Hierarchy of immunosuppressive strength among myeloid-derived suppressor cell subsets is determined by GM-CSF. Eur J Immunol *40*: 22-35, 2010.
35 Fridman WH, Pages F, Sautes-Fridman C and Galon J: The immune contexture in human tumours: impact on clinical outcome. Nat Rev Cancer *12*: 298-306, 2012.
36 Knutson KL and Disis ML: Tumor antigen-specific T helper cells in cancer immunity and immunotherapy. Cancer immunology, immunotherapy: CII *54*: 721-728, 2005.
37 Yu YA, Zhang Q and Szalay AA: Establishment and characterization of conditions required for tumor colonization by intravenously delivered bacteria. Biotechnol Bioeng *100*: 567-578, 2008.
38 Tome Y, Zhang Y, Momiyama M, Maehara H, Kanaya F, Tomita K, Tsuchiya H, Bouvet M, Hoffman RM and Zhao M: Primer dosing of *S. Typhimurium* A1-R potentiates tumor-targeting and efficacy in immunocompetent mice. Anticancer Res *33*: 97-102, 2013.
39 Schreiber K, Rowley DA, Riethmuller G and Schreiber H: Cancer immunotherapy and preclinical studies: Why we are not wasting our time with animal experiments. Hematology-Oncology Clinics of North America *20*: 567-584, 2006.
40 Peters LJ and Hewitt HB: The influence of fibrin formation on the transplantability of murine tumour cells: implications for the mechanism of the Revesz effect. Br J Cancer *29*: 279-291, 1974.
41 Lee JC, Kim DC, Gee MS, Saunders HM, Sehgal CM, Feldman MD, Ross SR and Lee WM: Interleukin-12 inhibits angiogenesis and growth of transplanted but not *in situ* mouse mammary tumor virus-induced mammary carcinomas. Cancer Res *62*: 747-755, 2002.
42 Greifenberg V, Ribechini E, Rossner S and Lutz MB: Myeloid-derived suppressor cell activation by combined LPS and IFN-gamma treatment impairs DC development. Eur J Immunol *39*: 2865-2876, 2009.
43 Ray P, Arora M, Poe SL and Ray A: Lung myeloid-derived suppressor cells and regulation of inflammation. Immunol Res *50*: 153-158, 2011.
44 Tam JW, Kullas AL, Mena P, Bliska JB and van der Velden AW: CD11b+ Ly6Chi Ly6G- immature myeloid cells recruited in response to *Salmonella* enterica serovar Typhimurium infection exhibit protective and immunosuppressive properties. Infect Immun *82*: 2606-2614, 2014.

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