

Safety and immunogenicity of *Salmonella* typhimurium expressing C-terminal truncated human IL-2 in a murine model

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Abstract: *Salmonella enterica* serovar Typhimurium preferentially colonizes tumors *in vivo* and has proven to be an effective biologic vector. The attenuated *S. enterica* Typhimurium strain χ 4550 was engineered to express truncated human interleukin-2 and renamed *SalpIL2*. Previously, we observed that a single oral administration of *SalpIL2* reduced tumor number and volume, while significantly increasing local and systemic natural killer (NK) cell populations in an experimental metastasis model. Here we report that in nontumor-bearing mice, a single oral dose of *SalpIL2* resulted in increased splenic cytotoxic T and NK cell populations that returned to control levels by 4 weeks post oral administration. Though *SalpIL2* was detected in mouse tissues for up to 10 weeks, no prolonged alterations in peripheral blood serum chemistry or complete blood cell counts were observed. Similarly, comparative histopathological analysis of tissues revealed no significant increase in pyogranulomas in *SalpIL2*-treated animals with respect to saline controls. In *Rag-1* knockout mice, which have severely impaired B and T cell function, *SalpIL2* reduced growth of hepatic metastases. Furthermore, *SalpIL2* altered expression of several proinflammatory cytokines and chemokines in the serum of mice with pulmonary osteosarcoma metastases. These data further suggest that *SalpIL2* is avirulent and induces a cell-mediated antitumor response.

Keywords: *Salmonella* Typhimurium, natural killer cells, interleukin-2

Introduction

Patients diagnosed with distant metastases of colorectal adenocarcinoma and osteosarcoma have 11% and 30% 3-year survival rates, respectively.^{1,2} In contrast, patients with localized osteosarcoma or colon cancer achieve nearly 70% 3-year and 90% 5-year survival, respectively.^{1,3} Therefore, development of early detection techniques and more efficient tumor-targeting therapies are needed to improve survival in patients with metastatic tumors.

Salmonella enterica serovar Typhimurium is a Gram-negative intracellular facultative anaerobe transmitted via the fecal-oral route. After oral ingestion, *Salmonella* invades the intestinal epithelia, colonizes Peyer's patches, and enters the lymphatics and bloodstream by infecting inactivated macrophage and dendritic cells. The bacteria then escapes to systemic tissues including the liver, spleen, and lungs. In addition, attenuated *S. enterica* preferentially invade and multiply within tumors relative to normal tissue.⁴⁻⁶ We engineered an attenuated strain of *S. enterica* Typhimurium, χ 4550, to express a truncated human interleukin-2 (IL-2) protein and named it *SalpIL2*. A single oral dose of *SalpIL2*, in animals with established pulmonary osteosarcoma metastases, significantly reduced tumor burden while increasing pulmonary natural killer (NK) cell

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populations as compared to non-IL2-expressing *S. enterica* and saline controls.⁷ Furthermore, *SalpIL2* oral administration prior to tumor injection prevented the establishment of metastatic tumors and increased splenic and hepatic NK cell populations.^{8,9} Although *SalpIL2* tracks to established tumors, it is unknown whether its antitumor effect is completely immune cell-mediated or is directly toxic to tumor cells *in vivo*.

Here we seek to further characterize the antitumor effect of a single oral administration of *SalpIL2* in experimental mouse tumor models. In a murine infection model, *SalpIL2* was avirulent and immunogenic, and within 16 weeks after oral administration colony-forming units (CFU) were no longer detectable in nontumor tissues, including the spleen, liver and lungs. Oral administration of *SalpIL2* resulted in a maximal increase of splenic NK cell populations within 14 days and decreased metastatic hepatic tumor burden in a T cell-independent manner. Lastly, oral administration of *SalpIL2* in tumor-burdened animals significantly altered the abundance of several proinflammatory serum cytokines. These data strongly suggest that *SalpIL2* is highly immunogenic, reduces tumor burden and tumor-associated systemic inflammation, while increasing proliferation of tumor-targeting cytotoxic NK cells.

Materials and methods

Tumor cells

Mouse osteosarcoma K7M2 (ATCC no. CRL-2836) and colon adenocarcinoma MCA-38 cells were maintained and prepared as previously described.^{7,9} All cultured cells were used within 18 passages from the master cell bank and were greater than 90% viable as determined by trypan blue exclusion.

Bacteria

The attenuated *S. enterica* Typhimurium strain χ 4550 was a kind gift from Dr. Roy Curtiss III (Washington University, St. Louis, MO). Plasmid constructs with or without a truncated C-terminal human interleukin-2 gene were electroporated into χ 4550 and the resulting strains were named *SalpIL2* and *Sal-NG*, respectively.

Animals

Bacterial administration

All animals were orally administered 200 μ L of Hank's buffered salt solution (HBSS) or HBSS containing 2×10^8 CFU *Sal-NG* or *SalpIL2* and monitored for 15 minutes to ensure the bacteria were not aspirated. All mice were housed

under the strict care of the University of Minnesota Research Animal Resources in a biosafety level 2 facility.

Biodistribution

Six to eight-week-old female C57BL/6 mice were gavaged either saline or *SalpIL2* or *Sal-NG*. Mice were harvested weekly for 4 weeks to determine tissue toxicity of *SalpIL2* or *Sal-NG*. In an additional experiment, 110 16 to 20-week-old female C57BL/6 mice were randomly separated and orally administered either saline or *SalpIL2* and sacrificed as described below.

Harvest procedure

On days 3, 7, 14 and biweekly until week 16, randomly selected groups of ($n = 5$) saline and *SalpIL2*-treated mice were anesthetized with a ketamine/xylazine (1:2) combination. Animal tissues were aseptically harvested and separated for determination of *S. enterica* CFU per gram of tissue or placed in 10% formalin and submitted for independent pathology analysis. The tissues analyzed included the heart, thymus, brain, lung, liver, spleen, stomach, small intestine, large intestine, bone marrow, intra-abdominal lymph nodes and kidneys. Formalin-fixed tissues were examined for pathological signs of *Salmonella* infection or IL-2 toxicity by Charles River Laboratories, Pathology Associates, Maryland (PAI). For determination of *S. enterica* CFU, half of each aforementioned tissue was weighed and mechanically minced to a homogenous slurry in 4°C phosphate-buffered saline (PBS) with sterile glass stoppers. Bone marrow was collected by aseptic removal and perfusion of the femur with sterile PBS. In addition, urine and feces were aseptically collected from the bladder and large intestine. All tissues and bodily fluids were cultured in triplicate on MacConkey II and Hektoen agar plates overnight.

Serum collection

Peripheral blood at all experimental endpoints collected from the right atrium of anesthetized animals was analyzed for complete blood count (CBC), centrifuged for serum chemistries or stored at -20°C for cytokine analysis.

Serum chemistries

Glucose, aspartate aminotransferase (AST), alanine transaminase (ALT), alkaline phosphatase (Alk Φ), bilirubin, cholesterol, total protein (TP), albumin, globulin, blood urea nitrogen (BUN), creatinine, phosphorous, calcium, sodium, potassium, chloride, bicarbonate, anion gap and gamma-glutamyl transferase (gamma-GT) serum concentrations were examined by the University of

Minnesota's Research Animal Resources' in-house veterinary laboratory.

Complete blood cell counts

White blood cells (WBC), neutrophils (NE), lymphocytes (LY), monocytes (MO), eosinophils (EO), basophils (BA), red blood cells (RBC), mean corpuscular volume (MCV), hemoglobin (Hb), hematocrit (HCT), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), red blood cell distribution width (RDW), platelets (PLT), mean platelet volume (MPV), packed cell volume (PCV), and total plasma protein (TPP) were analyzed by the University of Minnesota's Research Animal Resources' in-house veterinary laboratory.

PCR analysis of *Salmonella* colonies

Colonies morphologically similar to *S. enterica* Typhimurium were confirmed to be *SalpIL2* by polymerase chain reaction (PCR) for the presence of the plasmid containing truncated interleukin-2 sequence. The forward primer 5'-TGAATTGGAAGATCTCCGG-3' anneals 127 nt upstream from the transcription start site of the truncated IL-2 and the reverse primer 5'-GTTTTGGCGGATGAGAGAAG-3' anneals 4 nt downstream from the truncating stop codon resulting in a PCR product of 587 bp. PCR products were run on 2% agarose gels against no template controls and known *SalpIL2* stock as controls to ensure maintenance of plasmid *in vivo* and identification of the genotype.

Splenic lymphocyte response to *Salmonella*

In duplicate experiments, 100 6 to 8-week-old C57BL/6 female mice were orally administered saline, *Sal-NG*, or *SalpIL2* as described above. Cohorts of animals ($n = 5$) were sacrificed 3, 7, 14, 21 and 28 days after oral gavage for collection of splenic mononuclear cells. Lymphocytes were stained with rat antimouse conjugated antibody (BD Pharmingen, San Diego, CA) using previously described methods and identified with a FACScalibur flow cytometer (Becton Dickinson, Grenoble, France) and analyzed with Cell Quest Pro Software (Becton Dickinson, San Jose, CA).⁹ Lymphocyte populations were identified using forward scatter versus side scatter profiles and gated for mononuclear lymphocytes. NK, CD8⁺ and CD4⁺ cell populations were identified by single-positive populations based on 10,000-gated events.

Tumor metastasis models

Six to eight-week-old female Balb/c and *Rag-1*^{-/-} mice were acquired from Harlan Sprague Dawley (Indianapolis, IN). Mice were orally gavaged 7 days prior (prophylactic model)

or 3 days after tumor injection (treatment model) with standardized bacterial stocks as previously described.⁷ In triplicate experiments, 2×10^5 K7M2 osteosarcoma cells were injected in the left lateral tail vein. Additional mice were sacrificed for hematoxylin and eosin (H&E) analysis of animals in prophylactic and treatment osteosarcoma models. Aseptically removed lungs were submitted for H&E analysis by the University of Minnesota's Histopathology core. In the hepatic metastasis model, mice were injected with 5×10^4 MCA-38 colorectal cancer cells as previously described.⁹ Mice were separated into the following groups: 2 saline controls (no tumor injection), 5 C57BL/6 controls, 9 *Rag-1*^{-/-} saline controls, 8 *Rag-1*^{-/-} *Sal-NG*, and 10 *Rag-1*^{-/-} *SalpIL2*.

Cytokine analysis

Serum was collected from mice with K7M2 pulmonary metastasis at the time of sacrifice. Samples were loaded into a 96-well plate and analyzed following manufacturer's guidelines with a Bio-Plex 23-Murine Cytokine Assay (Bio-Rad, Hercules, CA) on the Bio-Plex 200 Array Reader.

Statistical analysis

Statistical tests were performed with StatView software (v. 5.0.1; SAS Institute, Cary, NC). CFU/cell and serum cytokines in osteosarcoma experiments and hepatic lymphocyte populations and tumor volume in *Rag-1*^{-/-} mice were entered for each specimen at the experimental endpoint to calculate the total mean values for each treatment group. Statistical significance between groups were determined by Fisher's exact test. Values from splenic lymphocytes, CBC and serum chemistries were entered for each mouse at the experimental endpoint to calculate the total mean values for each treatment group. Statistical significance between groups was determined by Students' unpaired *t*-test for serum and CBC and Fisher's exact test for splenic lymphocytes.

Results

Biodistribution of *SalpIL2*

C57BL/6, in contrast to Balb/c mice, have an exaggerated Th-1 cell-mediated immune response and is the genetic background for many transgenic knockout animals including the *Rag-1*^{-/-} T-cell knockout strain.^{10,11} Thus, C57BL/6 is a model strain for examining the virulence of an attenuated intracellular pathogen. *Salmonella* infection appears to be age dependent in C57BL/6 mice, as animals under 24 weeks are significantly more resistant to infection than their aged counterparts.¹² In this study, 6 to 8-week-old C57BL/6 mice orally administered *Sal-NG* and *SalpIL2* showed no

Table 1 CFU per gram tissue of a single oral gavage of 10^8 CFU of *SalpIL2* in naïve 16- to 20-week-old C57BL/6 mice. Results are mean values from animals with *SalpIL2* positive CFU. Numbers in parentheses represent the number of animals out of five with detectable *SalpIL2* in the respective tissue. *SalpIL2* was not detected in tissue from control animals at any harvest time

Time	2 h	3 d	1 w	2 w	4 w	6 w	8 w	10 w	12 w	14 w	16 w
Thymus	2.67 (1)	–	–	–	3.41 (1)	–	–	–	–	–	–
Brain	–	–	3.04 (1)	3.09 (1)	2.65 (1)	–	–	–	–	–	–
Stomach	2.99 (3)	–	–	–	–	2.24 (1)	–	2.95 (1)	–	–	–
S Intestine	3.82 (2)	3.76 (1)	–	–	–	–	–	–	–	–	–
L Intestine	4.30 (2)	–	–	–	–	–	–	–	–	–	–
Lymph	2.28 (2)	–	3.34 (1)	2.99 (2)	3.12 (3)	3.93 (1)	–	3.97 (1)	–	–	–
Lung	1.74 (1)	4.51 (2)	3.34 (3)	3.56 (1)	2.93 (3)	2.22 (1)	–	–	–	–	–
Heart	–	2.24 (1)	–	3.45 (1)	–	–	–	–	–	–	–
Kidney	2.29 (1)	–	–	–	–	1.97 (1)	–	2.49 (1)	–	–	–
Liver	1.68 (2)	2.68 (3)	2.21 (4)	2.67 (2)	2.41 (4)	2.29 (1)	–	2.01 (1)	–	–	–
Spleen	2.38 (1)	3.26 (3)	3.39 (5)	4.06 (4)	4.14 (5)	3.40 (3)	2.87 (3)	3.11 (1)	–	–	–

Abbreviations: CFU, colony-forming units; h, hours; d, days; w, weeks.

significant differences in the presence of pyogranulomas, CBC or serum chemistries up to 4 weeks following inoculation (data not shown). We next examined the biodistribution and toxicity of *SalpIL2* in 16 to 20-week-old C57BL/6 mice compared to saline controls over 16 weeks (Table 1). *SalpIL2* CFU were recovered from the liver and spleen as early as 2 hours post oral administration and detected in the spleen of all treated animals at the 1-week sacrifice. Detectable *SalpIL2* colonies remained high until week 4, after which there was a progressive decrease in the number of positive mice. Except for CFU in the stomach of one animal at weeks 6 and 10, *SalpIL2* was cleared from the digestive tract within 1 week. We did not detect *SalpIL2* in the bone marrow, urine or blood at any time point during the experiment and it was only observed in the fecal material in a single animal during the first 14 days (data not shown). Animals tested positive for *SalpIL2* in the lung for up to 6 weeks, and an increasing number of *SalpIL2* CFUs were observed in the lung over time. Acute CFU concentrations decreased in gastrointestinal tract tissue within one week of oral infection, but remained elevated in organs with major capillary beds (lung, liver, and spleen) or with significant immune involvement (thymus and intra-abdominal lymph nodes). Detection of CFUs in the heart and brain were limited to only one mouse per time point. Except for the intra-abdominal lymph nodes, *SalpIL2* CFU concentrations decreased in all tissues by week 6 and CFU were not detected in any animal after week 10.

Serum and CBC

Oral administration of *SalpIL2* increased serum chloride concentration compared to saline controls within 2 hours (115.33 mmol/L vs 110.75 mmol/L respectively; $P < 0.05$)

(Figure 1). By day 3, mean platelet volume (MPV) decreased in *SalpIL2*-treated animals relative to controls (4.78 fL vs 5.50 fL; $P < 0.01$), and the number of platelets (PLT) increased (1053 vs 615; $P < 0.05$) (Figure 3). Also by day 3, in animals treated with *SalpIL2* vs controls: EO increased (0.054 K/ μ L vs 0.002 K/ μ L; $P < 0.05$), MO increased (0.204 K/ μ L vs 0.090 K/ μ L; $P < 0.05$), NE increased (0.604 K/ μ L vs 0.210 K/ μ L; $P < 0.0209$), and WBCs increased (3.854 K/ μ L vs 2.132 K/ μ L; $P < 0.0284$) (Figure 2). By week 4, RDW significantly decreased with *SalpIL2* treatment (0.189% to 0.202%; $P < 0.05$). Although RDW values in *SalpIL2*-treated animals did not vary between weeks 4 and 6, control values decreased 20% and by week 6 *SalpIL2*-treated animals had significantly increased RDW relative to controls (0.194% vs 0.180%; $P < 0.01$) (Figure 3). Also in week 6, in animals treated with *SalpIL2*, MCV decreased (40.2 fL vs 42.7 fL; $P < 0.01$) (Figure 3), and the amount of Alk ϕ increased (96.25 U/L vs 84.33 U/L; $P < 0.05$) (Figure 1). In week 8, *SalpIL2* treated animals had decreased MCV (39.06 fL vs 41.92 fL; $P < 0.05$) (Figure 3) and increased phosphorous (8.3 mg/dL vs 5.5 mg/dL; $P < 0.05$) (Figure 1). At week 14, decreases were observed in creatinine (0.100 mg/dL vs 0.175 mg/dL; $P < 0.05$) and BUN (20 vs 28; $P < 0.01$) (Figure 1). Differences in remaining serum and blood chemistries examined were determined to be statistically insignificant.

Histopathology

Microscopic histopathological analysis revealed that the liver in *SalpIL2*-treated animals underwent the most changes of all tissues examined. Pyogranulomas defined as aggregations of neutrophils, macrophages and mononuclear

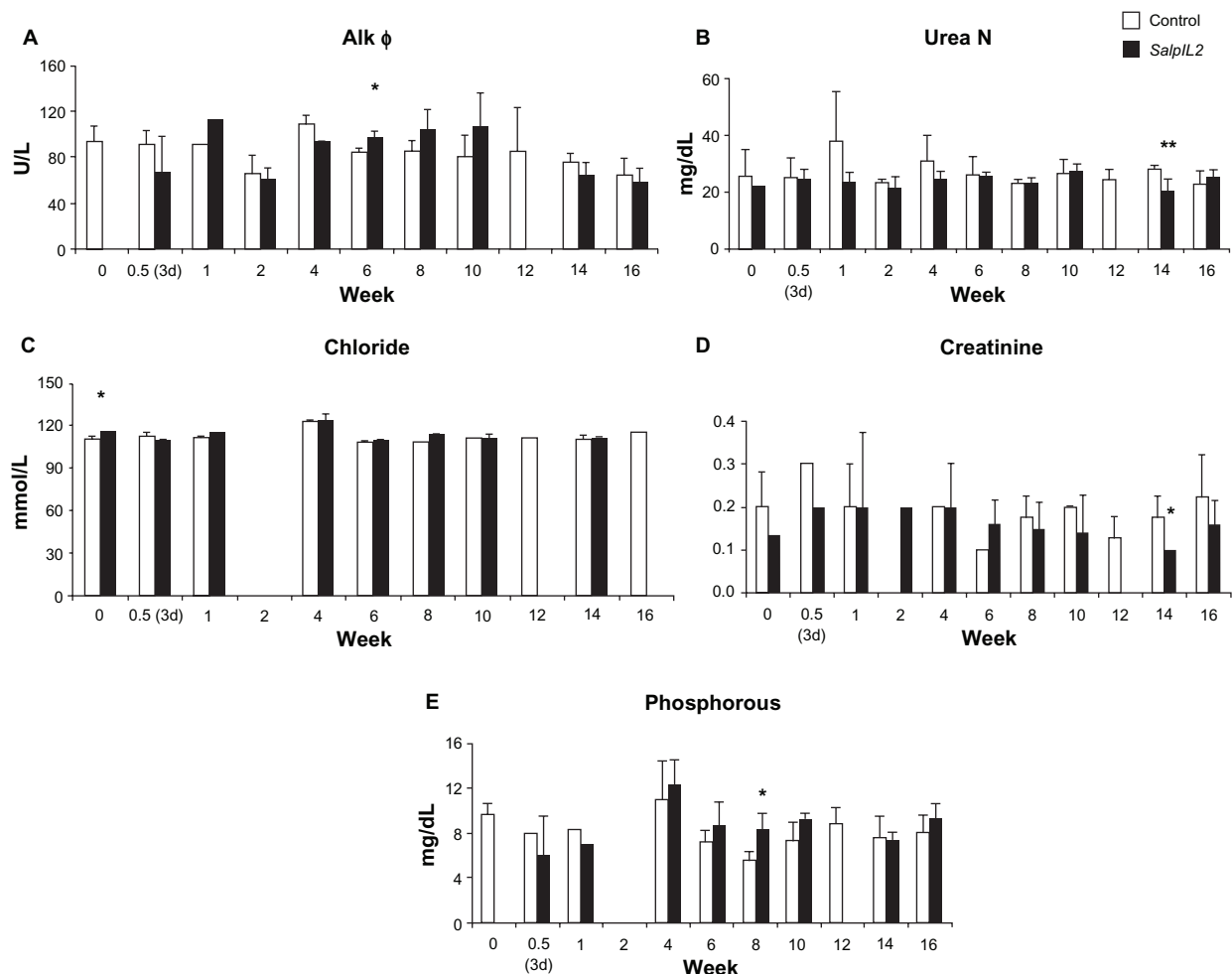


Figure 1 The effect of a single oral administration of *SalpIL2* on mouse serum chemistry. Asterisks indicate statistically significant changes in serum chemistry as determined by unpaired Student's *t*-test. Results presented are mean values from groups of five *SalpIL2*-treated and five saline control animals.

Notes: Error bars represent one standard deviation. Alkaline phosphatase is denoted as Alk ϕ . **P* < 0.05, ***P* < 0.01.

lymphocytes were present in the liver at 3 and 7 days post oral gavage in two of five mice and classified as mild to moderate. At week 2, three of five livers had mild to moderate pyogranulomas and by week 4 all livers had multifocal pyogranulomas that were classified as mild (1 of 5) or moderate (4 of 5). At 6 and 8 weeks, four of five livers in each treatment group had multifocal pyogranulomas graded as minimal or mild. At 10 weeks, three of five livers in the *SalpIL2* group had mild to moderate pyogranulomas. *SalpIL2*-treated mice at weeks 12 and 14 had minimal (focal) pyogranulomas with one liver at each time point having multifocal lesions. By week 16, all pyogranulomas in *SalpIL2*-treated livers were graded as mild or were no longer present in treated mice. In contrast, the saline-treated animals had minimal to mild pyogranulomas in the liver at 6, 10, 12 and 16-week sacrifices. Acute Kupffer cell hyperplasia was detected in as many as four of five *SalpIL2*-treated mice at 1, 2, and 4 weeks post oral gavage and one of five livers

had the increase at weeks 6, 8, and 10. No control animals had Kupffer cell hyperplasia.

Spleens from *SalpIL2*-treated animals displayed increased myelopoiesis from day 3 to week 4 after oral administration, followed by a steady decrease from weeks 6 through 14. Erythropoiesis was observed in tissues from *SalpIL2*-treated animals collected at 2 hours and on day 14. Although a rare occurrence, spleens of control animals showed increased erythropoiesis on day 3 and contained multiple mild pyogranulomas at week 4. Similarly, one control lung sample had moderate pyogranulomas present on day 3 and one animal in the 4- and 8-week sacrifices had minimal pulmonary pyogranulomas accompanied by increased pulmonary neutrophils.

Bone marrow tissue displayed mildly increased myelopoiesis as soon as 3 days after administration, moderate myelopoiesis by 2 weeks and mild to obvious myelopoiesis at 4 weeks. Minimal myelopoiesis was present at 6 and 8 weeks in treated animals and continued to decrease until week 14.

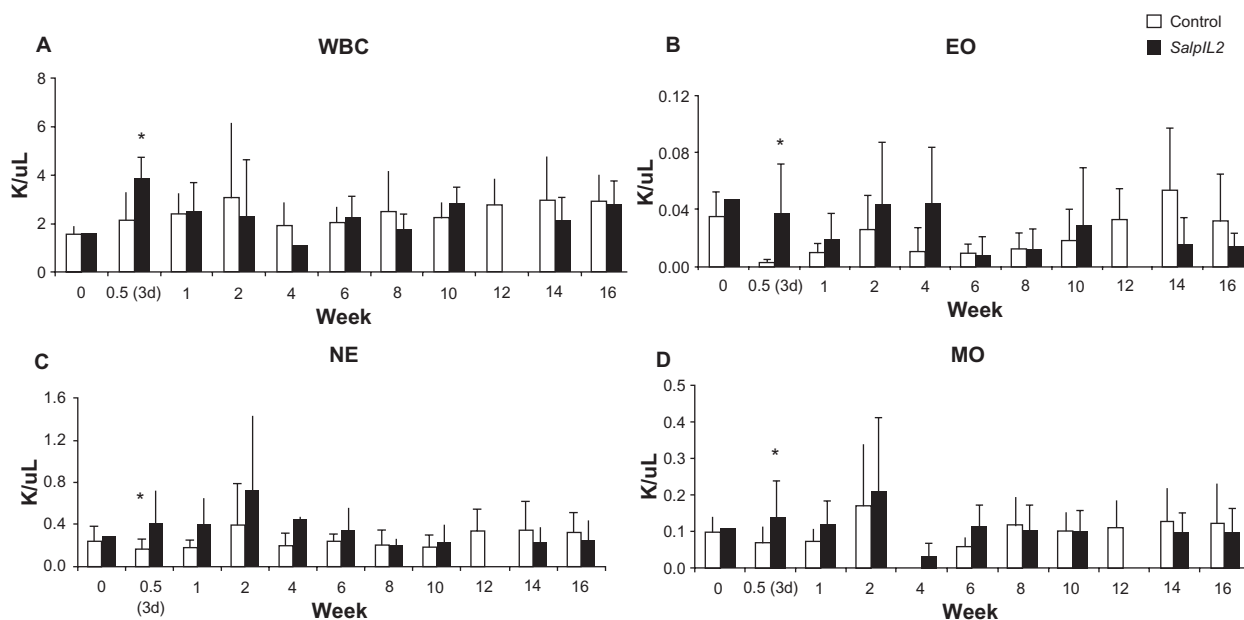


Figure 2 The effect of a single oral administration of *SalpIL2* on complete blood cell counts. Asterisks indicate differences determined to be statistically significant by unpaired Student's t-test. White blood cells (WBC), neutrophils (NE), monocytes (MO), eosinophils (EO). Results presented are mean values from groups of five *SalpIL2*-treated and five saline control animals.

Notes: Error bars represent one standard deviation. * $P < 0.05$.

In one control animal at 10 weeks, there was marked histolytic cell infiltration of the bone marrow. Minimal to moderate acute inflammation in the submucosa was observed in the stomachs of the *SalpIL2*-treated animals. In contrast, control animals showed decreased inflammation in the

stomach during this period, and inflammation was absent in all animals by week 12. Intra-abdominal lymph nodes had minimal to moderate increased reticuloendothelial cells in the majority of treated mice at weeks 6 and 8, and in 1 treated animal at all other time points. Four control animals also

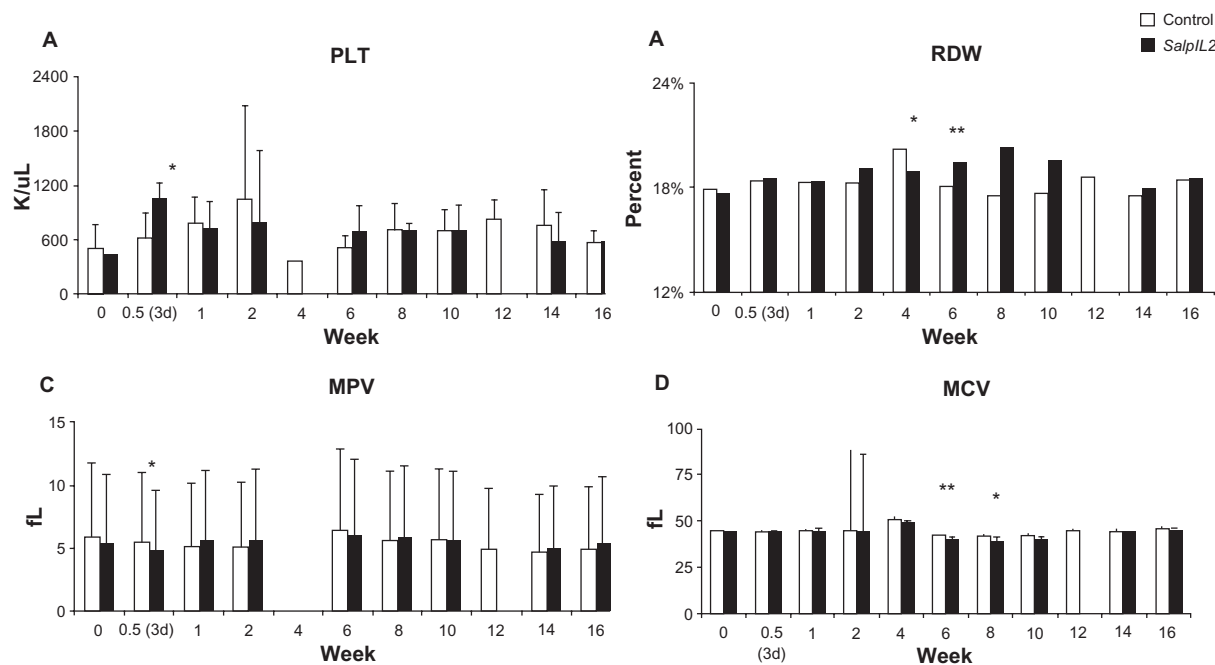


Figure 3 The effect of a single oral administration of *SalpIL2* on blood cell phenotype. Mean corpuscular volume (MCV), red blood cell distribution width (RDW), platelet (PLT) and mean platelet volume (MPV) average values are from groups of five *SalpIL2*-treated and five control animals per time point.

Notes: Error bars represent one standard deviation. Differences determined to be statistically significant by unpaired Student's t-test are indicated by asterisks. * $P < 0.05$, ** $P < 0.01$.

had reticuloendothelial cell hyperplasia. In the brain, two *SalpIL2*-treated mice in the 4-week group had mild, multifocal, lymphocytic infiltrates in the meninges. One of these mice had minimal cerebral cortical lymphocytic perivascular cuffing. Microscopic lesions were not observed in the thymus, intestines, heart, or kidney in *SalpIL2*-treated animals.

Splenic lymphocyte response to *SalpIL2*

Animals orally administered *SalpIL2* had an increased splenic NK 1.1⁺ cell population by day 7 which remained elevated on days 14 ($P < 0.01$) and 21 and returned to basal level by day 28 (Figure 4A). A significant increase in CD8⁺ T cells ($P < 0.001$) on day 3 was observed followed by a return to baseline by day 7 (Figure 4B). *SalpIL2* did not significantly alter the proportion of CD4⁺ T cells over the 28-day period (Figure 4C).

Hepatic metastasis model in *Rag-I*^{-/-}

To elucidate the role of cytotoxic T cells in the *SalpIL2* antitumor response, we used *Rag-I*^{-/-} mice, which do not have mature B or T cells, in an experimental hepatic metastasis model. A single oral dose of *Sal-NG* or *SalpIL2* significantly decreased tumor volume with respect to saline controls ($P < 0.001$; Figure 5A). Furthermore, compared to saline controls and *Sal-NG*, *SalpIL2* significantly increased hepatic NK cell populations detected by flow cytometry ($P < 0.001$ and $P < 0.05$ respectively; Figure 5B).

Serum cytokine analysis in experimental osteosarcoma treatment model

The K7M2 murine osteosarcoma cell line was derived and characterized from spontaneous pulmonary metastases in Balb/c mice.¹³ Using an experimental lung metastasis model

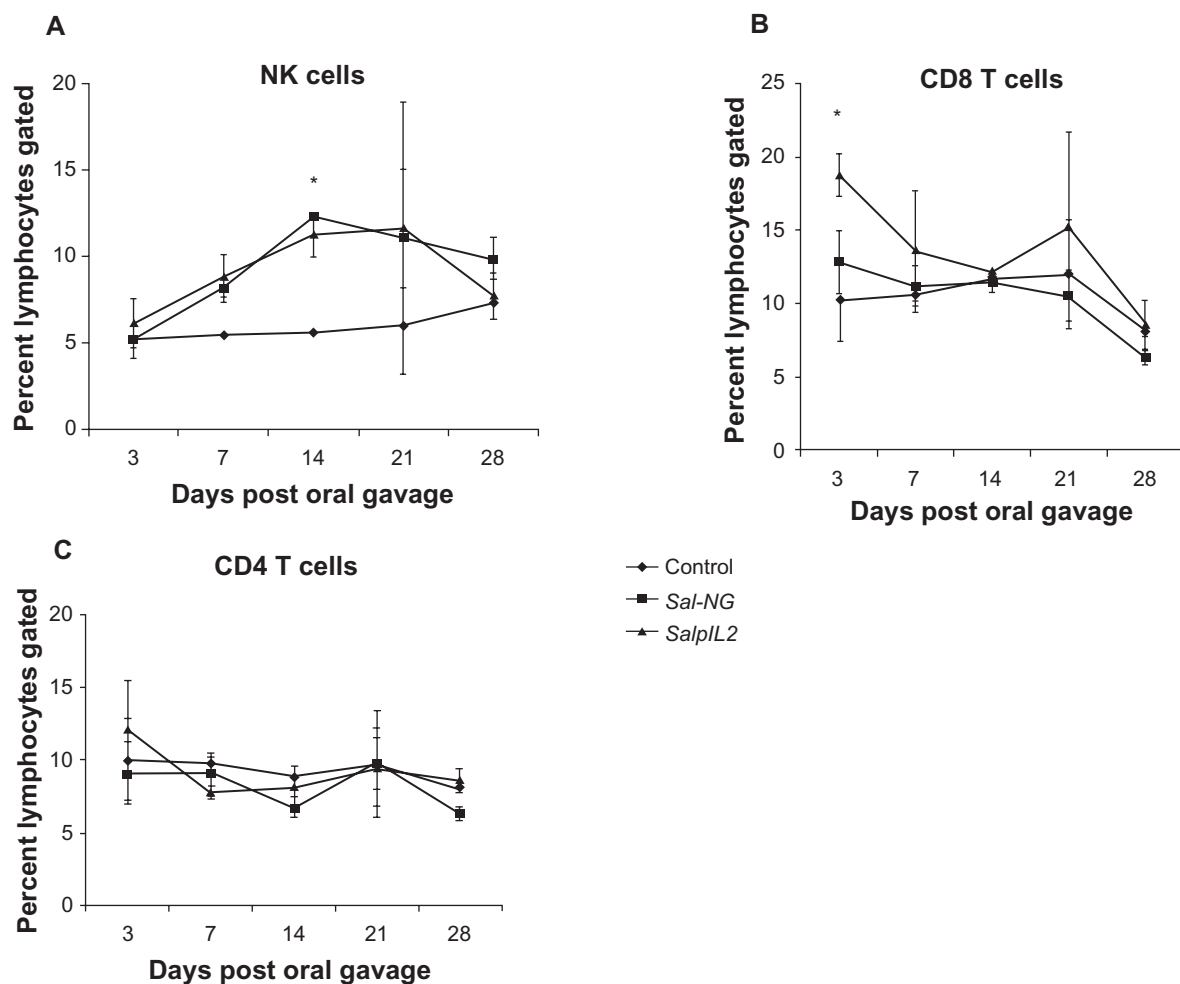


Figure 4 Natural killer (NK) cells and cytotoxic T lymphocytes are significantly increased in the acute systemic immune response to *SalpIL2*. NK cells (NK 1.1) and cytotoxic T cells (CD8⁺) populations are significantly increased in response to *SalpIL2*. Helper T cells (CD4⁺) populations are not significantly affected by a single oral administration of 2×10^8 CFU of *SalpIL2* or *Sal-NG*.

Notes: Results from triplicate experiments presented as $\pm$ standard error of mean, were determined to be statistically significant by unpaired Fisher's exact test and indicated by asterisks. * $P < 0.05$.

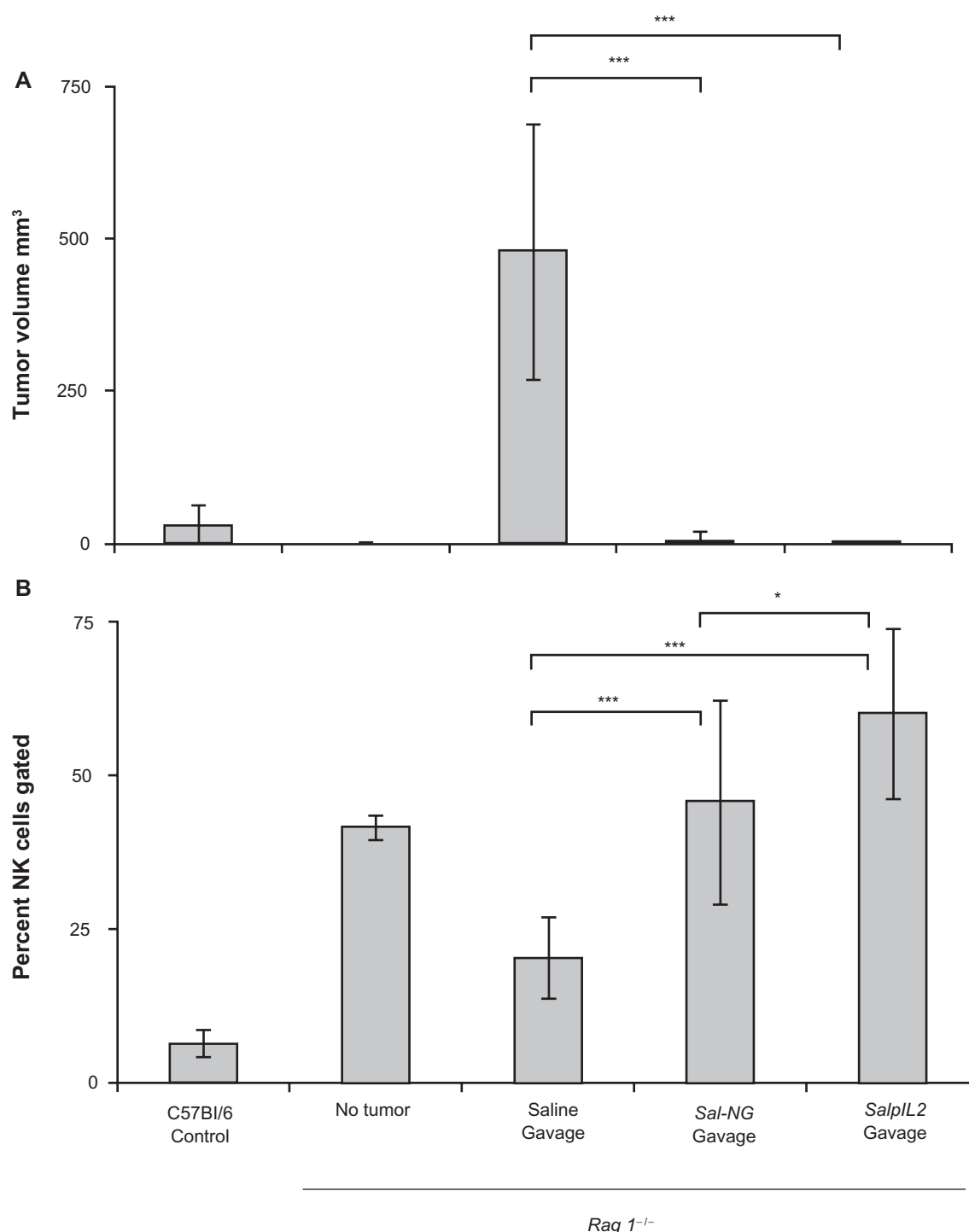


Figure 5 *SalpIL2* antitumor effect is independent of cytotoxic T lymphocytes. *SalpIL2* increases NK cell population and reduces the macroscopic volume of hepatic metastases in *Rag1^{-/-}* mice, which have significantly suppressed T lymphocyte populations.

Notes: Results from one representative experiment shown, $N = 7$. * $P < 0.05$, *** $P < 0.001$.

that produces macroscopic lesions isolated to the lung, we analyzed sera from animals at 3-weeks post tumor injection. Serum cytokines in the treatment model were largely not significantly affected by oral administration of *SalpIL2* or *Sal-NG*. Granulocyte colony-stimulating factor (G-CSF) was

significantly increased from a control mean of 45.7 $\mu\text{g/mL}$ to 107.7 $\mu\text{g/mL}$ ($P < 0.05$) in *Sal-NG* and 114.1 $\mu\text{g/mL}$ ($P < 0.05$) in *SalpIL2* (Figure 6C). In addition, *Sal-NG* treated mice displayed 65% increase in IL-12p40 ($P < 0.05$) whereas *SalpIL2* treated mice did not display a significant

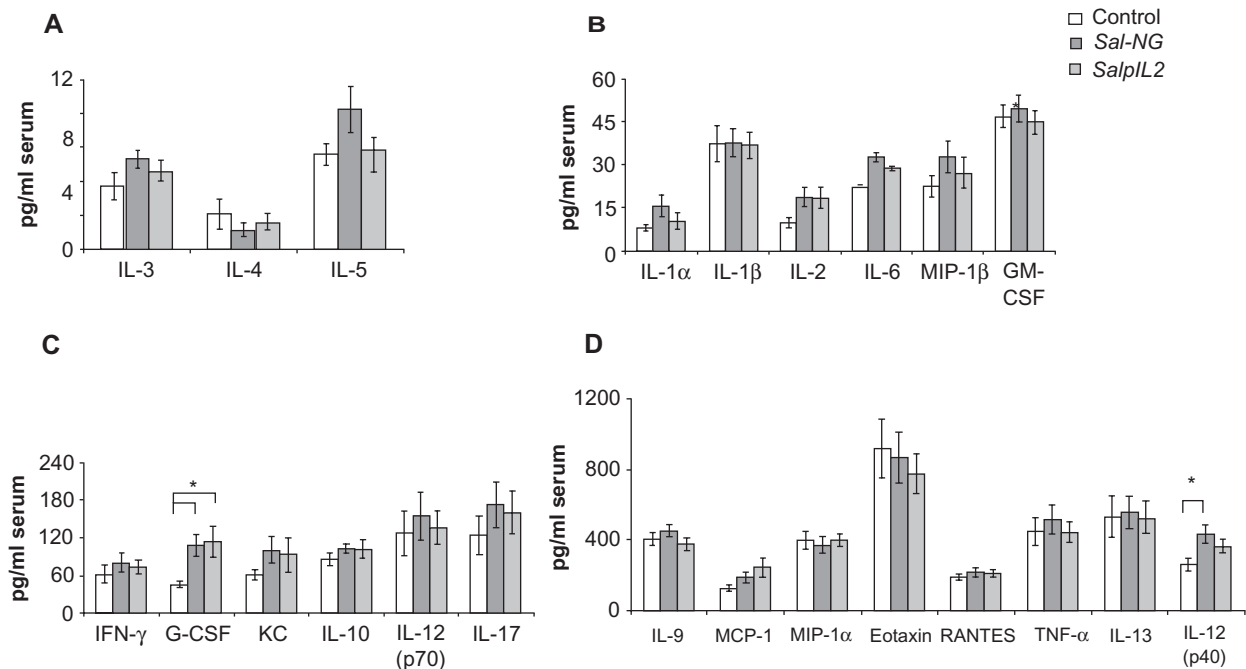


Figure 6 Oral inoculation of *Salmonella* alters serum cytokines during treatment of metastatic osteosarcoma. *SalpIL2*, but not *Sal-NG* significantly increases serum G-CSF with respect to saline controls ($P < 0.05$ and $P = 0.276$, respectively).

Notes: Results presented as SEM from triplicate experiments. * $p < 0.05$.

Abbreviation: G-CSF, granulocyte colony-stimulating factor.

increase in IL-12p40 (Figure 6D). Systemic IL-2 was not significantly increased with *SalpIL2* or *Sal-NG* oral gavage with respect to saline controls (Figure 6B).

Serum cytokine analysis in experimental prophylactic model

In the prophylactic model of pulmonary metastasis, several systemic cytokines were significantly lower in *Sal-NG* and *SalpIL2* groups than in saline controls. *Sal-NG* significantly lowered IL-1 β ($P < 0.01$), KC ($P < 0.0257$), and MCP-1 ($P < 0.001$) with respect to control animals (Figure 7). *SalpIL2* significantly decreased systemic IL-1 β ($P < 0.001$), IL-3 ($P < 0.0346$), KC ($P < 0.01$), MCP-1 ($P < 0.001$), MIP-1 β ($P < 0.05$), and RANTES ($P < 0.05$) compared to saline controls.

Discussion

Salmonella enterica serovar Typhimurium is a Gram-negative intracellular facultative anaerobe transmitted via the fecal-oral route. Upon oral administration, *Salmonella* migrate to the gastrointestinal tract and colonize lymphoid sections of the small intestine, called Peyer's patches. In addition, intraepithelial dendritic cells provide a route for *Salmonella* to gain access to underlying tissues and other innate immune cells, thus circumventing the barrier provided

by the intestinal epithelium.¹⁴ *Salmonella*-infected dendritic cells then migrate to the mesenteric lymph nodes for antigen processing.¹⁵ Apoptosis of infected macrophage and dendritic cells during their progression through the liver and spleen result in preferential colonization of these organs.¹⁶ Recent evidence suggests *S. enterica*'s survival in hepatic phagocytes includes the formation of a foci, release by necrosis or apoptosis, and invasion of surrounding cells that results in a new foci from a single bacteria.^{17,18} *S. enterica*'s colonization of major organs and tissues may persist for up to a period of 4 to 6 months as a result of its ability to reside within an endosome as a facultative intracellular bacteria.¹⁹ *S. enterica* Typhimurium is an attractive delivery vector for intra-tumoral expression of cytokines or antigenic molecules from viruses, bacteria, and some parasites in various animal models.^{20–26} Methods of *Salmonella* administration in cancer models varying from the oral route to intra-tumor or intravenous injections have been shown to be efficacious.^{27–29} During our investigations we have examined the safety and efficacy of a single oral dose of the attenuated *S. enterica* Typhimurium χ 4550 in reducing tumor burden in mice. The χ 4550 strain is an adenylate cyclase, cyclic adenosine monophosphate receptor protein and aspartate semialdehyde dehydrogenase deletion mutant that does not express a functional flagella.³⁰ Oral administration of *Salmonella* may be

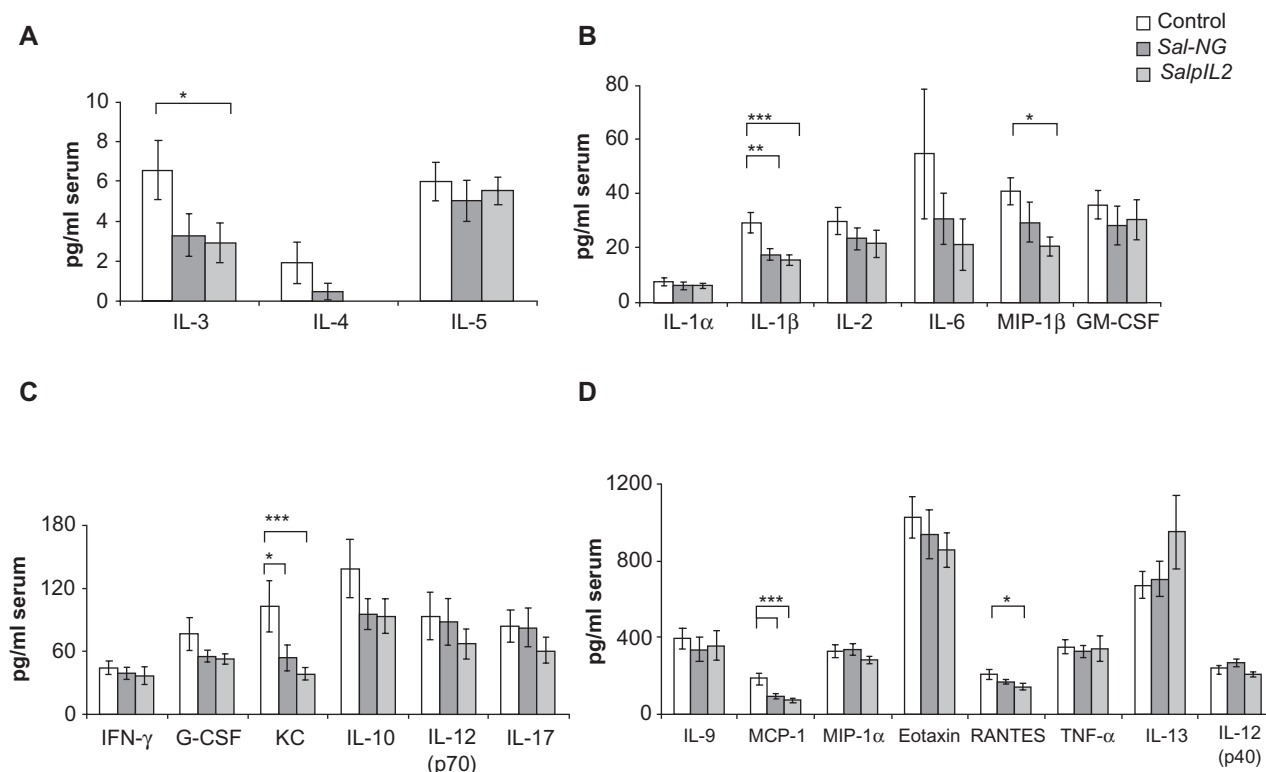


Figure 7 Oral inoculation of *Salmonella* alters serum cytokines during the prevention of metastatic osteosarcoma. *Sal-NG* significantly lowers IL-1β ($P < 0.01$), KC ($P < 0.05$) and MCP-1 ($P < 0.001$) with respect to control animals. *SalpIL2* significantly decreases systemic IL-1β ($P < 0.001$), IL-3 ($P < 0.05$), KC ($P < 0.01$), MCP-1 ($P < 0.001$), MIP-1β ($P < 0.05$), and RANTES ($P < 0.05$) as compared to saline controls.

Notes: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

advantageous over intravenous delivery by allowing for use of standardized bacterial glycerol stocks and stimulation of both the systemic and mucosal immunity against intracellular pathogens. As argued by Belyakov and Ahlers, activating intraepithelial dendritic cells at mucosal surfaces produces a more robust response to intracellular pathogens including *Salmonella*.³¹ We have chosen to examine the safety and efficacy of a single oral dose in our models to reduce the potential inhibitory effects of the secretory IgA immune response against multiple *Salmonella* administrations.³² In prior investigations, a single oral dose of $\chi 4550$ engineered to express a human IL-2 activated NK cells against metastatic hepatic malignancies.^{9,26,33}

Here we examined the antitumor mechanism of *S. enterica* expressing a C-terminal truncated human IL-2 gene. Supporting the pathological finding of a mild inflammatory response to a single oral administration of *SalpIL2*, splenic mononuclear lymphocytes were increased significantly within the first 7 days of oral administration, but returned to levels similar to control animals by week 4. Furthermore, oral *SalpIL2* inoculation resulted in sporadic

increases of inflammatory cells determined by CBC that gradually decreased as the study progressed. The MPV was decreased following *SalpIL2* administration, while the total number of platelets present in the blood nearly doubled, likely due to hematopoietic stimulation in the spleen and bone marrow. White blood cells, eosinophils, monocytes and neutrophils were all increased in the first 2 weeks consistent with concurrent infection. Recovery of *SalpIL2* CFU was largely restricted to the liver and spleen and analysis of serum chemistries demonstrated no difference in liver or spleen function between control and *SalpIL2*-treated animals throughout the experiment. Some evidence of hepatic stress was expected as evidenced by the rise in Alk ϕ . Slight increases in Alk ϕ at the week 6 sacrifice and normal bilirubin throughout demonstrate the ability of the infected animal to maintain normal liver enzymatic function despite mild hepatitis resulting from *SalpIL2* infection. Acute increases in serum chloride were perceived to be a result of the gavage media. Renal function was determined to be unaffected as *SalpIL2* administration resulted in only a slight decrease in serum BUN and creatinine concentrations at week 14 and a

slight increase of phosphorous at week 8. In some animals the volume of blood collected was not sufficient to examine all of the possible serum chemistry. Therefore some chemistry was unable to be examined at every sacrifice time point. Despite this limitation, we had a sufficient number of mice per group to determine the general systemic affects of a single oral dose of *SalpIL2*. Most importantly no *SalpIL2*-treated animal had detectable CFUs after 10 weeks of infection or displayed outward signs of morbidity, demonstrating the ability of the bacteria to be cleared without antibiotic treatment.

Based on these results, *SalpIL2* did not produce a virulent disease state in mice. We were unable to demonstrate morbidity associated with mild hepatitis, or any significant organ dysfunction. The treatment and control animals maintained food consumption within normal established limits. Although a few *SalpIL2*-treated mice developed meningitis and cerebritis, this observation was isolated to a single cage. It is unclear whether gavage technique or cage conditions may have resulted in *SalpIL2* infection of the central nervous system in those animals. Although the natural mode of *Salmonella* infection is fecal-oral, we were only able to culture *SalpIL2* from the stool in one mouse over all time points. This may be a result of the inocula load or attenuation of *SalpIL2*.

Oral administration of *SalpIL2* did not create detectable systemic toxicity that may be cytotoxic to tumors in mice at any time point. Moderate increases in systemic NK and acute CD8⁺ T cell populations strongly suggest *SalpIL2*'s antitumor mechanism is cell-mediated tumor toxicity. As we reported previously, in a pulmonary metastasis model *SalpIL2*, but not *Sal-NG*, significantly increased NK but not CD8⁺ T cells in the lung relative to saline controls.⁷ Furthermore, depletion of liver NK cells and CD8⁺ T cells, but not CD4⁺ T cells and Kupffer cells abrogated the antitumor effect of *S. enterica* species.²⁶ Taken together, these data would suggest that although both *SalpIL2* and *Sal-NG* can induce systemic NK cell proliferation, *SalpIL2* is more efficient at attracting NK cells to distant tissues to direct an antitumor response. Because *S. enterica* preferentially tracks to and multiplies within tumors, biodistribution of *SalpIL2* in tumor bearing animals may be augmented. Persistence of *SalpIL2* in tumors may prolong colonization of other tissues. However, mortality of animals with tumor significantly hinders any prolonged study on the differences in tumor-dependent *SalpIL2* distribution.

To further elucidate the role of the immune system in *SalpIL2*'s antitumor response, we used *Rag-1* deficient mice that have severely depressed B and T cells, including

tumor-supportive regulatory T cell populations. NK cells lacking rearranged T cell receptors develop in the bone marrow but function and develop normally in *Rag1* deficient mice.¹¹ Both *Salmonella*-treated groups significantly decreased tumor volume in a B and T-cell independent manner. *SalpIL2*, however, had significantly higher hepatic NK cell populations as compared to *Sal-NG* and saline controls. In animals with established pulmonary metastases, *Salmonella*-treatment increased expression of G-CSF, a chemokine that promotes monocyte activation and migration into tissues. These data suggest a strong cytotoxic immune response in the treatment animals. Most evident, in mice where tumors were introduced after *Salmonella* was administered, expression of several proinflammatory chemokines was significantly reduced. However, these observations may be due to the decreased tumor burden in the animal and may not be reflective of the local cytokine environment of the tumor and warrant further study.

S. enterica Typhimurium tumor vaccines have become more prevalent in recent years. Many investigators have determined the efficacy of their vaccines through the injection of *S. enterica* Typhimurium directly into the tumor or through intravenous delivery.^{34,35} Administration of these *S. enterica* strains has been encouragingly efficacious in murine primary tumor models. *S. enterica* antigen vaccines, however, are dependant on homogenous tumor expression of the antigen to be effective against metastatic tumors.²⁷ Investigators have shown intravenous administration of attenuated *S. enterica* to be safe in primates and humans in phase I clinical trials.^{36,37} In our investigations, oral administration of *SalpIL2* not only localized to the liver, spleen and pulmonary tissues, but also elicited a robust local and systemic NK cell response that was independent of tumor type or animal strain. In our pulmonary metastases model, osteosarcoma metastases formed clearly defined nodules and linear aggregates of invasive tumor cells centered around the pleura.⁷ Mononuclear infiltrate in *SalpIL2*-treated animals was positively correlated with a reduction in tumor burden and interpreted to represent tumor regression. Hence, the presence of mononuclear cell infiltrates appears to be an integral factor in the reduction of *SalpIL2*-treated tumors *in vivo*. Although oral administration induced a mild inflammatory response, these data strongly suggest *SalpIL2* is avirulent, immunogenic, and induces a robust NK cell-mediated antitumor response.

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BS, AL, and DS are stockholders in Botanic Oil Innovation. All other authors certify that he or she have no commercial associations that may pose a conflict of interest in connection with this manuscript.

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