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Salmonella Bacterial Monotherapy Reduces Autochthonous Prostate Tumor Burden in the TRAMP Mouse Model

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Abstract

Attenuated *Salmonella typhimurium* injected in the circulatory system of mammals selectively targets tumors. Using weekly intraperitoneal injections of attenuated *Salmonella* strain CRC2631, we tested for regression and/or inhibition of tumor development in the TRAMP prostate tumor mouse model, which utilizes SV40 early region expression for autochthonous formation of prostate tumors that progress into metastatic, poorly differentiated prostatic carcinomas in an immunocompetent murine model. Thirteen weekly intraperitoneal administrations of 10^5–10^7 CFU CRC2631 into 10 week old mice were well tolerated by the TRAMP model. Sacrifice and histological analysis of TRAMP prostates at 22 weeks indicated that *Salmonella* monotherapy at administrated levels decrease visible tumor size (>29%) but did not significantly inhibit previously described SV40 expression-driven TRAMP tumor progression to undifferentiated carcinomas when histologically examined. In conclusion, this work demonstrates baseline results for CRC2631 *Salmonella* monotherapy using the immunocompetent TRAMP prostate tumor model in preparation for study of combination therapies that resolve autochthonously generated TRAMP prostate tumors, further reduce tumor size, or inhibit prostate tumor progression.

Introduction

Combatting advanced and metastatic tumors is still one of the most difficult cancer treatment challenges and new therapeutic approaches are necessary to target this heterogeneous disease in which different cancer cells may require different treatments. Targeting the entire cancer cell population within solid tumors is a goal that may be achievable using attenuated bacterial strains, specifically *Salmonella enterica* serovar Typhimurium (*Salmonella*) that preferentially target and infiltrate tumors without affecting non-cancerous cells and tissue [1–3].

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Previous research has shown the feasibility of this approach [4–8]. However, high-dosage monotherapy with attenuated *Salmonella* strains proved too toxic and resulted in patients not tolerating the high amounts of attenuated *Salmonella* strains used in clinical studies [9]. These early results indicated that strain modifications as well as determining tolerable doses of *Salmonella* are essential for utilizing this promising therapy to control cancer. Due to direct tumor targeting by *Salmonella* it will also be possible to apply combination therapies by which a drug or cancer-destroying component is directly carried into the cancer cells by *Salmonella* for direct chemotherapeutic administration.

The idea of using bacteriotherapy for treatment of cancer was originally proposed more than a century ago, when heat-killed bacteria and their components were found to have the potential to inhibit cancer growth [10]. In the mid-1900s, it was observed that some bacteria had the ability to survive and replicate in hypoxic tumor tissues. In the last two decades, investigation of bacterial based tumor therapy (bacteriotherapy) has progressed rapidly. Bacterial species including *Salmonella* [11–14], *Listeria* [15, 16], and *Clostridium* [17, 18] have tumor targeting and tumor-destroying phenotypes that are being actively exploited for detection of and chemotherapeutic delivery to tumors [19].

Attenuated *Salmonella* candidates have been extensively studied for targeted treatment of cancer [11]. *Salmonella* are gram-negative facultative anaerobic bacteria that can grow and replicate inside host cells. *Salmonella* strains preferentially infiltrate and colonize solid tumor masses [2, 8] including autochthonous primary or implanted orthologous tumors in the prostate [20], lymph nodes [21], pancreas [22], breast [23], lung [24] and brain tissues [25, 26]. Although the mechanism(s) of *Salmonella* tumor colonization have not been fully elucidated, the *Salmonella* pathogenicity island 2 (SPI-2) is required for rapid amplification of *Salmonella* in tumor host cells [27, 28] that leads to tumor growth suppression [29, 30]. *Salmonella* genetic tools are robust and attenuated strains can be engineered to carry and/or synthesize chemotherapeutic payloads. Finally, *Salmonella* is an adjuvant that can assist in immunogenic recognition [27] and subsequent destruction of tumors [31–33], especially when used in combination with vaccines [34, 35]. The single phase I human clinical trial of a *Salmonella* strain (VNP20009) in human patients had excessive toxicity when used as a cancer monotherapy at high administration levels [9]. Subsequently, research on *Salmonella* as a bacteriotherapeutic has focused on engineering *Salmonella* strains with lowered toxicity [36] while preserving their unique tumor targeting and infiltration phenotypes [3, 37–39]. Delivery of chemotherapeutic payloads is designed to further reduce the *Salmonella* load needed for clinical effect and complete resolution of tumors.

Several *Salmonella* strains are being actively developed as bacteriotherapeutic vectors including VNP20009 [1], A1-R [23], SL7207 [40], LVR01 [41], and CRC2631 [6]. We have developed the tumor-targeting *Salmonella* strain model and candidate therapeutic (CRC2631) that is derived from the *Salmonella typhimurium* LT2 wild type [14]. The parental strain (CRC1674) was stored in agar stabs under nutrient-limiting conditions for more than four decades at room temperature, generating dramatic genetic diversity including deletions, duplications, frameshifts, inversions and transpositions [42, 43]. Genetic investigation indicates CRC1674 contains numerous mutations: originally an LT2 *his*-2550 strain, CRC1674 acquired a *his* suppressor mutation, DIIR49B, an altered *rpoS* start signal (UUG), G to T mutation in position 168 in *rpoS* sequence, and decreased HPI and HPII [14]. CRC1674 was further engineered to disrupt *aroA*, *thyA*, and *rfaH* to generate an LPS-deficient strain auxotrophic for biosynthesis of aromatic amino acids and thymine. The resulting attenuated strain, CRC2631, did not change its tumor targeting and tumor cell destruction phenotype but decreased its toxicity dramatically. Co-incubation of CRC2631 and human prostate cancer cell line PC-3M results in colonization of PC-3M and destruction of their mitochondria within one hour [6]. Up to

1.2×10^8 CFU of CRC2631 can be tolerated in TRAMP mice (an immunocompetent autochthonous prostate cancer model), showing its safety in mammalian hosts. When intraperitoneally injected with $1{\times}10^7$ CFU, the ratio of *Salmonella* counts were up to 100-fold greater in the TRAMP mouse prostate tumor masses versus the usual *Salmonella* reservoirs of the liver and spleen after 72 hours. We have performed morphologic and phenotypic analysis of CRC2631 species recovered from TRAMP mouse prostate tumors to evaluate the selective pressures of cancer targeting and persistence in the novel tumor environment [44].

In order to investigate the ability of CRC2631 to serve as a chemotherapeutic carrying vector, we have explored its effect as a monotherapy in the TRAMP mouse, an autochthonous prostate cancer model triggered by testosterone driven SV40 large and small T-antigen expression [45]. The TRAMP prostate cancer model was chosen due to its autochthonous tumor generation, well-characterized tumor progression stages, and immunocompetency [46]. Male TRAMP mouse prostate tumor progression from 8–24 weeks of age proceeds from spontaneous prostatic intraepithelial neoplasia (PIN), to well-differentiated carcinomas (WDC), Phylloides-like lesions (PHY), and finally poorly differentiated carcinoma (PDC) [47–49]. In addition to testing for increased survival time and tumor size inhibition, measuring inhibition of tumor progression of the TRAMP prostate tumor model is also possible; TRAMP prostate tumors have been partially inhibited from progressing to the late stage WDC and PDC development by up to 81% using plant-derived botanical compounds that have been shown to inhibit the Hedgehog signal pathway [50] as well as limited inhibition of WDC incidence when fed the phytoestrogen genistein [49]. This demonstrates that the TRAMP model is excellent for relatively rapid analysis of primary tumor inhibition at varying mono- or multivalent therapy dosages with simultaneous analysis of adverse immunological effects.

In this paper, we report the effect of weekly intraperitoneal *Salmonella* injections on TRAMP mouse survival, tumor size, and progression. Groups of male TRAMP mice positive for SV40 antigen expression were intraperitoneally injected with 10^5–10^7 of *Salmonella* strain CRC2631 or a control buffer injection weekly from 10–22 weeks of age. Survival curve analysis was performed during this injection period. After the 22^{nd} week, surviving TRAMP models were sacrificed and the urogenital tracts extracted to measure visible tumor volumes and perform histological grading of prostate and any visible tumors. Our results show that increasing CRC2631 *Salmonella* monotherapy is well tolerated in the TRAMP model and when given during the 10–22 week tumor development window, decreases the size of visible prostate-associated tumors although under our current experimental conditions it does not prevent tumor progression in the prostate tumor model. This study shows that the TRAMP model is excellent for studying the effect of *Salmonella*-mediated cancer targeted combination therapies including delivery of cancer-inhibiting molecules, generation of anti-cancer peptides or triggering immunostimulatory reactions at the tumor site using *Salmonella*-mediated cancer targeting.

Materials and Methods

Bacterial Strain Culturing and Preparation

Salmonella strain CRC2631 (derived from nutrient-limited LT2 auxotroph CRC1674 [51]) was used in this study. See Table 1 for complete strain information. All *Salmonella* were grown on nutrient Luria-Bertani (LB) agar plates (25g/L LB powder (Fisher BioReagents), 15 g/L agar (Fisher BioReagents) in deionized water) supplemented with 200 μg/mL thymine (Acros Organics) at 37°C overnight. Strains were cultured in liquid medium by stab inoculating 10mL LB broth (25g/L LB powder in deionized water supplemented with 200 μg/mL thymine in sterile 50mL tubes (Thermo Scientific) with isolated colonies and incubating in a 37°C dry shaker for 16–20 hours.

Table 1. Bacterial Strains and Animal Models.

Organism	Genotype	Reference
Salmonella enterica serovar Typhimurium		
CRC1674	LT2 *hisD*2550 *rpoS*	Sutton et al (2000)
CRC2631	CRC1674 *aroA*::Tn10TcΔ*rfaH* Δ*thyA*::pKD4	Zhong et al (2008)
Mus musculus		
TRAMP	C57BL6/J-PBTag+	Greenberg et al (1995), Sluzarz et al (2010)

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Overnight cultures of *Salmonella* CRC2631 grown for injection into mouse models were washed with sterile PBS, normalized to 10^8 colony forming units (CFU)/mL and diluted appropriately to administer 10^5, 10^6, and 10^7 CFU in 100μl of sterile PBS. Samples were loaded in sterile 25G 1mL TB syringes (BD) and kept at 4°C until injection.

TRAMP Mouse Studies

All experiments utilized the TRansgenic Adenocarcinoma of Mouse Prostate (TRAMP) C57BL6/J-PBTag+ mouse model (Table 1), originally developed by using the prostate-specific rat probasin promoter (PB) to drive expression of the oncogenic simian virus 40 large tumor antigen-coding region (Tag) [45]. The autochthonous prostate tumor formation and progression in the TRAMP model is well established and considered suitable for use in prostate cancer studies of tumor progression and prevention [46] [49].

Male TRAMP mice were raised on site at the University of Missouri (Columbia, MO) as previously described [50]. University of Missouri institutional guidelines for animal care and use were followed. Mice were housed in pathogen-free microisolator-type cages with wood shaving bedding at 70–75°F, 35–65% humidity with a 12 hour day/night cycle. Mice were free-fed with 5001 Laboratory Rodent diet (LabDiet) and water. Three different concentrations (10^5,10^6, and 10^7 CFU) of viable *Salmonella* (CRC2631) in 100μl PBS were intraperitoneally injected each week for 12 weeks into one of three groups of twenty TRAMP mice from 10–22 weeks of age. A fourth control group was intraperitoneally injected with 100μl sterile PBS as a negative control. Animals were euthanized at the study endpoint (end of 22nd week) following University of Missouri Animal Care and Use Committee standard operating protocols. After euthanasia, the prostate and associated tumor masses were harvested, measured using a caliper, and immediately fixed in Shandon™ Formal-Fixx™ 10% Neutral Buffered Formalin (Thermo Scientific) overnight at 4°C before harvesting tissues for histological analysis. Two cross section samples (1-2mm) of each fixed prostate sample (or tumor mass if prostate was completely transformed) were paraffin embedded, sectioned (4μm thick sections), mounted on glass slides and stained with hematoxylin and eosin (H&E) for examination and tumor grading by light microscopy.

Animal Research and Welfare

All animal work was performed at the University of Missouri, Columbia, MO, USA. Experimental protocols and animal husbandry were approved by the University of Missouri Animal Care and Use Committee, Columbia, MO, USA (#7642). On injection days, mice were observed every 2 hours for 8 hours to check for unexpected acute reactions to dosage. On non-injection days, mice were examined daily. During observations a pain/distress evaluation was performed for each of the mice as recommended by the University of Missouri Standard Policy on Painful or Distressful Procedures (University of Missouri IACUC, July 2006) to detect signs of toxicity (e.g. no interest in cage exploration, excess Harderian gland secretions, loss of

coordination, over 10% body weight loss, loss of appetite, difficulty breathing). Mice with a score >1.0 on the toxicity and discomfort scale or exhibiting obvious signs of distress were taken and humanely euthanized (S1 File). Animals were euthanized (CO_2 inhalation for 10 minutes followed by cervical dislocation to ensure euthanasia) at the study endpoint (end of 22nd week) following University of Missouri Animal Care and Use Committee standard operating protocols. During the study, there was one unexpected combat death between mice. The remaining deaths in the study were due to TRAMP prostate tumor development (natural morbidity of the mouse line). On-site veterinarian staff administered analgesics as needed.

Histology

Each prostate was sampled twice. One tissue section per slide was viewed and graded. The veterinary pathologist was unaware of the duplicate slides or treatment groups until after grading. Dorsal prostate tubules were graded individually and placed into one of six categories: 1) normal tissue, 2) hyperplasia (HYP), 3) prostatic intraepithelial neoplasia (PIN), 4) well differentiated carcinoma (WDC), 5) phylloides-like (PHY) and 6) neuro-endocrine-like / poorly differentiated carcinoma (PDC) phenotype as previously described [49]. Identification of neuroendocrine-like poorly differentiated carcinoma (PDC) lesions caused a stage of PDC to be assigned to the animal regardless of the status of the dorsal prostate. Anterior prostate tubules were also viewed and recorded as displaying hyperplasia or well-differentiated adenocarcinoma but not specifically counted or graded. When appropriate, tumors were recorded as localized to a specific section of tissue (i.e. periurethral region) or as affecting the entire tissue.

Statistical Analysis

GraphPad Prism 6 was used to perform analyses (GraphPad, La Jolla, CA). We used the LIFETEST procedure in SAS 9.4 to compute nonparametric estimates of the survival functions and to compare the survival curves. This procedure was used since we had a high presence of right-censored data from terminating the experiment before many mice died in order to collect prostate tissue for histology. To test whether Salmonella injections significantly inhibited tumor progression, we used Fisher's exact test.

Results and Discussion

Weekly intraperitoneal *Salmonella* injections are well tolerated in the TRAMP mouse model

TRAMP mouse models of autochthonous prostate cancer groups (n = 20) were injected intraperitoneally with 10^5, 10^6, and 10^7 CFU of *Salmonella* strain CRC2631 in 100μl PBS. Intraperitoneal (IP) injections of 100μl PBS were performed in one group as a negative control. Survival curves were plotted during the study period (Fig 1). Survival curves indicate no significant change in survival for the TRAMP model over the study period at any injection level (S1 Fig, S1 Table). One mouse in the 10^7 group died from combat-associated injuries with cage mates; this death was excluded in the survival curve analysis because we cannot tell if the death was due to combat injuries or tumor burden.

Average size of tumor volumes in TRAMP model decrease with increased *Salmonella* injection levels

Prostate and prostate-associated tumors were extracted from surviving TRAMP mice at study endpoint. Volumes of prostate-associated tumor masses were measured using calipers (Fig 2). Mice with no visible tumor masses were not measured. Visible tumor mass mice dosed with

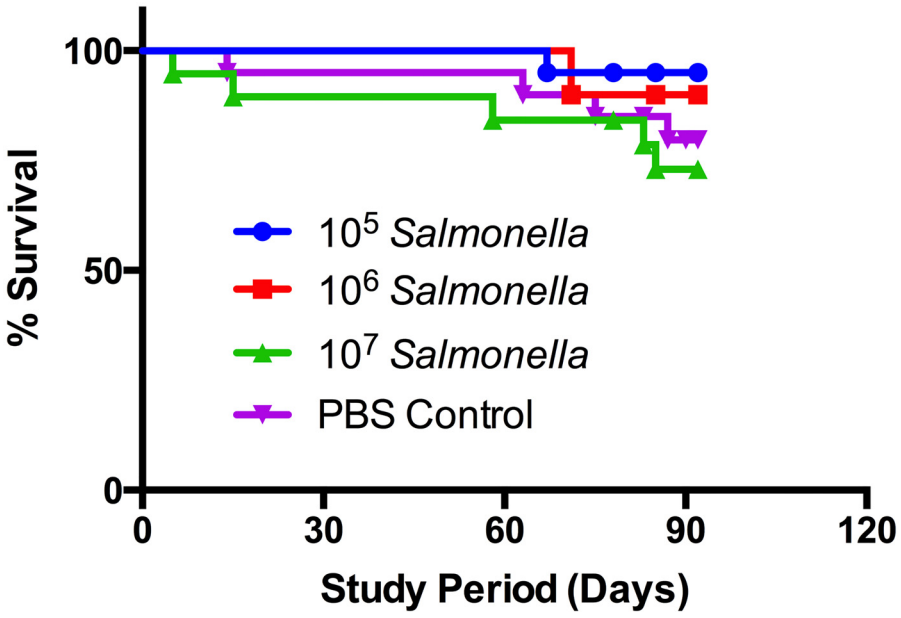

Fig 1. Weekly *Salmonella* injections tolerated by TRAMP mouse model. Survival curve of TRAMP mouse groups (n = 20) aged 10–22 weeks (98 days) during study period with weekly IP injection of 100μl PBS (control) or 100μl PBS with *Salmonella* at indicated concentrations. Surviving mice were sacrificed at the end of week 22.

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Salmonella had 29.2% smaller average tumor burdens. Average volume of recovered tumors decreased with increase in *Salmonella* dosage. Due to the single data point in the control group, this data is qualitative.

Histological grading of prostate tumors in the TRAMP mouse model

Prostates along with any associated tumors were extracted from the surviving TRAMP mouse models and fixed overnight in 10% buffered formalin at 4°C. Two cross sections of the dorsal

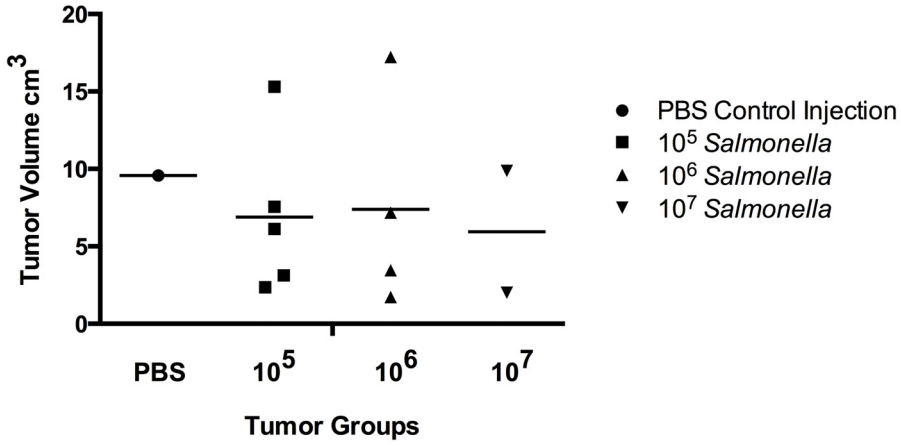

Fig 2. Tumor volume decreases with increasing *Salmonella* dosage. Caliper measurement of prostate-associated tumor volumes extracted from TRAMP mouse groups at end of study with visible excess tumor growth. Mean tumor volumes: PBS control (n = 1/16 with visible excess growth) 9.57 cm^3, 10^5 injection group (n = 5/17 with visible excess growth) 7.45 cm^3, 10^6 injection group (n = 5/17 with visible excess growth) 6.43 cm^3, 10^7 injection group (n = 2/13 with visible excess growth) 5.94 cm^3.

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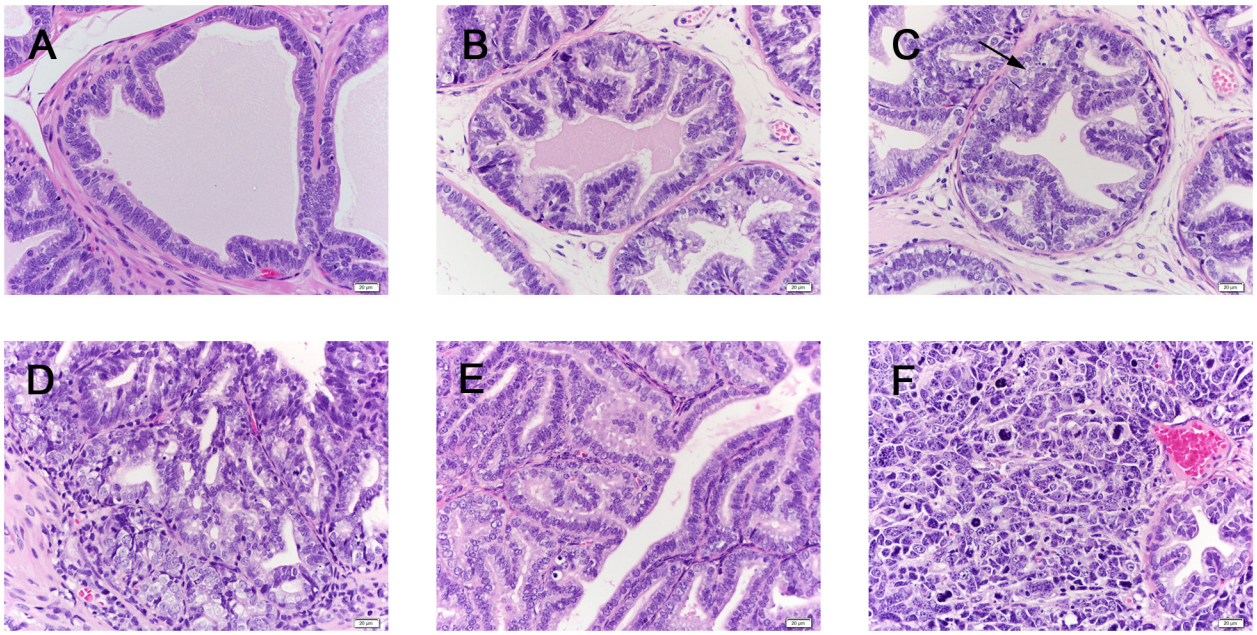

Fig 3. Histology of prostate tumor development in TRAMP mice. Histologic sections of the dorsal lobes of the prostate from transgenic mice stained with hematoxylin and eosin at 40X magnification. **Pathologic grades**: PIN, prostatic epithelial neoplasia; WD, well-differentiated adenocarcinoma; PHY, phylloides-like; PDC, poorly differentiated neuroendocrine-type carcinoma. **Slides**: (A) Normal tissue, (B) Hyperplastic tissue, (C) PIN, (D) WD, (E) PHY and (F) PDC (neuroendocrine-type). **Observations**: (C) Note tufting of epithelial cells, increased mitoses, hyperchromatic nuclei, stratification of nuclei and cribiform structures (arrow). (D) Note neoplastic cells with round nuclei; tumor type is characterized by increased numbers of small glands and thickening of the stroma. (E) Note staghorn luminal patterns of neoplastic cells. (F) Note the high nuclear:cytoplasmic ratio of neoplastic cells, loss of glandular differentiation and marked cell pleomorphism.

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prostate and any associated tumors were sampled by sectioning, hematoxylin and eosin staining, and grading using previously established criteria [49]. Histological grading of *Salmonella*-treated prostates ranged from normal tissue to poorly differentiated carcinomas (Fig 3). Comparing tumor stages individually, using Chi-square test for association for PDC and Fisher's exact test for PHY and WD (because some cell sizes are smaller than 5), The p-values were all non-significant: PDC p-value = 0.1725, PHY p-value = 0.3967, WD p-value = 0.4458. Overall association between the injection groups and tumor progression is not statistically significant (P-value = 0.3314) using Fisher's exact test. Therefore, *Salmonella* injections did not significantly inhibit tumor progression in the TRAMP prostate cancer model (Table 2). Histological observation indicated the presence of neuroendocrine-type tumors at the periurethral region in twelve TRAMP prostate samples (example, Fig 4). While neuroendocrine tumors in the TRAMP model have been previously reported to invade the periurethral region, this has previously been reported as always associated with a morphologically identical large tumor arising in the prostate [47]; we only observed large neuroendocrine tumors associated with two of the twelve samples.

Prostate cancer is still the second-leading cause of cancer-related deaths in men [52]. Advanced cancer treatment still represents medical challenges, as effective cures are still not yet available. While androgen deprivation therapy (ADT) is effective in treating early stages of advanced prostate cancer most patients respond to this treatment initially but their cancers become androgen-independent and most patients become ADT resistant [53, 54].

Furthermore, a surprising emergence of neuroendocrine prostate cancer cells (NEPC) has resulted from androgen deprivation therapy (ADT), which presents new challenges for

Table 2. Effect of weekly Salmonella dosages on prostate tumor development in the 5 month TRAMP prostate.

		Farthest tumor progression in prostate		
***Salmonella* Dosage**	**n**	**WD**	**PHY**	**PDC**
PBS Control	16	4	5	7
10^5 *Salmonella*	17	2	3	12
10^6 *Salmonella*	17	6	5	6
10^7 *Salmonella*	13	4	1	8

Numbers indicate the farthest stage of progression for each prostate sample. n = sample size. WD, well-differentiated adenocarcinoma; PHY, phylloides-like carcinoma; PDC, poorly differentiated neuroendocrine-type carcinoma.

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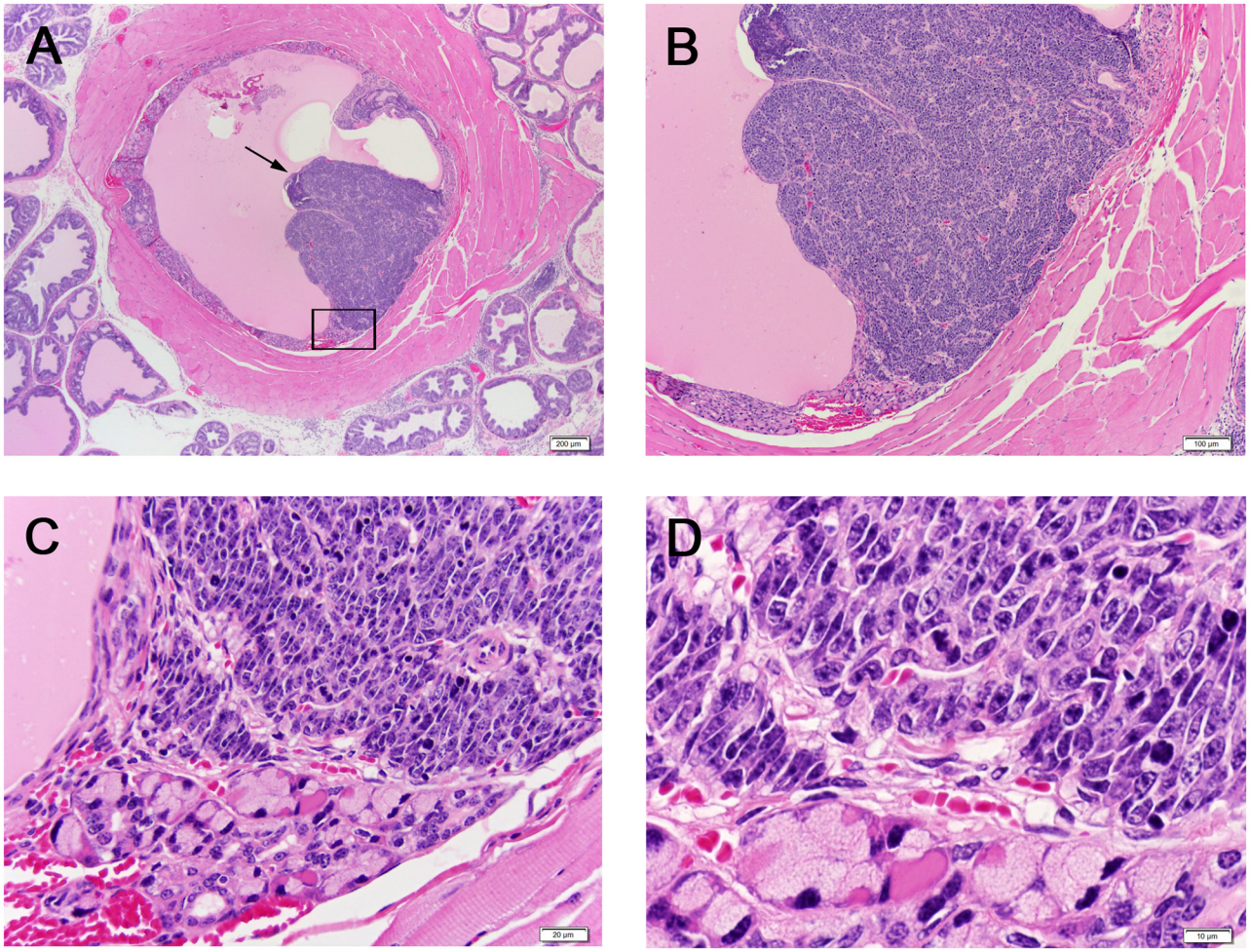

Fig 4. Neuroendocrine-type carcinoma in the periurethral region of a TRAMP mouse. Histologic sections of the periurethral region from a transgenic mouse stained with hematoxylin and eosin (H&E) at 4X (A), 10X (B), 40X (C) and 100X (D). (A): Note discrete tumor (arrow) within the epithelium of the periurethral region. The outline in (A) is the magnified region shown in (C) and (D).

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treatment. While the AR-negative neuroendocrine prostate cancers (NEPC) are rare at the time of initial diagnosis, they can account for 5–30% of advanced prostate cancers promoted by ADT [55].

Effective therapy for advanced stages of androgen-independent prostate cancer is still not yet available. Although progress has been made toward identifying the problems associated with disease progression, it has become clear that there is a need to eradicate the various subpopulations of this heterogeneous disease including the hard-to-treat and oftentimes radiation-resistant cancer stem cell (CSC) population. New therapies are critically needed to target these subpopulations that may require different and combination treatment strategies.

While rigorous and targeted therapies for the various subpopulations of prostate cancer might be developed in the distant future new treatment options aimed at targeting the entire cancer tissue with all subpopulations are currently in development and actively pursued in various laboratories using different approaches. A number of laboratories, including ours, are utilizing attenuated bacteria for targeting and chemotherapeutic activation/delivery ("bacteriotherapy") of cancers. These approaches are expected to have advantages over surgery and radiotherapy and will also eliminate newly observed cancer stem cell populations that have presented new challenges for treatment of cancer, as the inability to provide new treatment options is still associated with poor prognosis. The cancer stem cell subpopulation is responsible for prostate tumor initiation, recurrence, drug-resistance and metastatic progression. *Salmonella* targeting significantly reduces the weight of tumors initiated by cancer stem-like cells in several studies [56, 57].

Salmonella have been shown to effectively target and colonize any tumors that can be accessed by the host circulatory system, whether the *Salmonella* is introduced by intravenous, intraperitoneal, or oral delivery [11, 12]. *Salmonella* adapted to target tumors for detection have been predicted to colonize and detect tumor masses more than 2000-fold smaller than current tumor detection methods utilizing tomography [19].

Evolved to survive in mammalian hosts, *Salmonella* has the ability to adapt host membrane vesicles for its own use and can manipulate the placement of the membrane vesicle for replication and infiltration (invasion) of adjacent host cells [27]. As a facultative anaerobe, *Salmonella* can colonize both the oxygen-rich tumor periphery and anoxic tumor mass [8, 38, 58]. Once the entire tumor is colonized, *Salmonella* can deliver attached chemotherapies or synthesize molecules including cancer-killing chemotherapeutics, enzymes for activating drugs (prodrugs) at the cancer site [59, 60], transfer and/or express genes to inhibit cancer oncogene expression, or produce immune signaling molecules for cancer immunotherapy [35]. The high infiltration rate of *Salmonella* makes it superior to current nanoparticle technology that is limited in how far it can penetrate tumor tissue due to the high interstitial pressure characteristic of tumor masses [61]. Novel combination bacteriotherapies including targeted delivery of anticancer molecules (carried or synthesized), immunostimulatory peptides or vaccines, enzymes designed to activate prodrugs at tumors, and radiation [62] combination therapies, all concentrated at the tumor site using *Salmonella*, have been and continue to be actively researched by our laboratory and other laboratories in the field of cancer-targeting bacteriotherapy with increasing levels of success.

The single limitation to *Salmonella* bacteriotherapy is concern about potential toxicity seen in cancer patients using the VNP20009 *Salmonella* strain during phase I clinical testing in 2002 [9]. Since that study, efforts have been made by multiple laboratories to reduce toxicity in *Salmonella* without disrupting its cancer targeting, invasion, and tumor infiltration phenotypes. We engineered a novel, attenuated *Salmonella* bacteriotherapeutic strain (CRC2631) that is non-toxic and exhibits cancer targeting, invasion, and cancer cell destruction phenotypes [6]. Additionally, we have developed tools to facilitate *Salmonella* vector delivery of combination

chemotherapies in order to increase bacteriotherapeutic effectiveness and reduce the dose of *Salmonella* needed for clinical effect [63].

The effect of *Salmonella* monotherapy on prostate tumor progression in an immunocompetent model has not been characterized. In the present study, we examined the effect of weekly administration of our bacteriotherapeutic *Salmonella* strain (CRC2631) on prostate tumor progression. We used the TRAMP mouse model of prostate cancer that utilizes testosterone-driven expression of the SV40 large and small T-antigen [45] to generate autochthonous primary prostate tumors that eventually develop into poorly differentiated carcinomas with neuroendocrine carcinomas. Tumor progression in the TRAMP model is well documented [49, 50, 64] and provides an excellent model to test prostate tumor progression inhibition of *Salmonella* monotherapy and combination chemotherapies in an immunocompetent mammalian model that translates well to study prostate cancer progression in human patients.

We have shown that *Salmonella* CRC2631 injections in the immunocompetent TRAMP model are well tolerated; survival curves of TRAMP mice during the 10–22 week injection period show no significant decrease in survival in TRAMP mice during weekly IP injections of 10^5–10^7 CFUs of *Salmonella* versus control injections of sterile PBS. Secondly, the mean size of visible prostate-associated tumors observed in TRAMP mice decreased when *Salmonella* was administered; as more *Salmonella* was administered, the average size of prostate-associated tumors also decreased. Due to the single data point in the PBS control group (Fig 2) we cannot state with confidence that CRC2631 caused significant reduction of tumor size; however this qualitative data in combination with the reduction of TRAMP mice at the final PDC stage of SV40 expression-induced prostate cancer in the 10^6 group (Table 2) suggests that CRC2631 Salmonella monotherapy is reducing their tumor burden in the models with excess tumor growth and increasing their quality of life. These subtle but promising results with CRC2631 Salmonella monotherapy make the immunocompetent, autochthonous TRAMP prostate cancer progression model an excellent candidate for evaluating combination therapies, including but not limited to inhibitors of the Hedgehog signaling pathway which has previously shown partial inhibition of TRAMP tumor progression [50]. The conclusion that Salmonella bacteriotherapy requires additional carried and/or expressed anti-cancer molecules delivered by tumor-infiltrated *Salmonella* (combination therapy) is a commonly held opinion by prominent researchers in the field of bacteriotherapy [12, 13, 36, 65].

In humans and mice the normal prostate is composed of stromal and epithelial compartments. The epithelial compartment contains luminal epithelial cells, basal cells and a few scattered neuroendocrine (NE) cells. NE cells have epithelial, neural and endocrine features. They are not evenly distributed in the prostate and are most often found in the periurethral region and verumontanum (colliculus seminalis) in humans [66]. NE cells can also be found in prostate cancer, with increased numbers of these cells in tumors associated with poor prognosis [66].

In humans, the term neuroendocrine differentiation (NED) in prostate cancer (PC) refers to the presence of singly scattered NE cells or cells in small nests in typical prostatic adenocarcinomas [66]. Focal neuroendocrine differentiation is common in human prostatic adenocarcinoma [67]. NED is seen in >30% of prostate cancer and is associated with poor prognosis (high grade and high stage tumors) and androgen independence [68]. About 5–10% of prostatic adenocarcinomas contain large numbers of NE tumor cells, however, pure NE tumors in humans are rare as primary cancers [66].

As in human neuroendocrine PC, neuroendocrine carcinomas in TRAMP mice are associated with rapid growth and metastases and are highly lethal. However, while only a small percentage of human prostate tumors are primary NE cancers, TRAMP mice have a high incidence of neuroendocrine tumors arising in the prostate, which often metastasize to the

lymph nodes, lung and liver [67]. In mice, neuroendocrine carcinomas are similar to human neuroendocrine carcinomas in appearance and are characterized by cells with high nuclear: cytoplasmic ratio of neoplastic cells, granular cytoplasm, loss of glandular differentiation and marked cell pleomorphism. They are the most widely metastatic and aggressive mouse prostate cancer [67]. The TRAMP model is therefore also suitable to study NED.

Based on histology analysis and grading, we did not find a significant reduction in tumor progression in the TRAMP model using *Salmonella* monotherapy. However, we had an unexpected and novel finding. Histological observation indicated the presence of neuroendocrine-type tumors at the periurethral region in twelve TRAMP prostate thick sections. While neuroendocrine tumors in the TRAMP model have been previously reported to invade the periurethral region, this has previously been reported as always associated with a morphologically identical large tumor arising in the prostate [47]; we only observed large neuroendocrine tumors associated with two of the twelve samples. We are quite confident that there are no larger tumors in the rest of the ten prostates with neuroendocrine-type tumors at the periurethral region, which is a novel observation in the TRAMP model. However, we cannot eliminate the possibility that there are small neuroendocrine-type tumors in the prostate because we did not perform serial sections of the ten prostates that did not have visible large tumors. Invasion of neuroendocrine type tumor cells at the periurethral region in the TRAMP model is important, as it demonstrates the utility of this model to study neuroendocrine type tumor cells that have become an important aspect for the treatment of aggressive prostate tumors. As indicated above, in human prostate cancer androgen deprivation therapy (ADT) is commonly used for treatment of prostate cancer, which is associated with promoting the progression of androgen receptor (AR)-positive adenocarcinoma cells (AdPC) to AR negative neuroendocrine prostate cancer (NEPC) through neuroendocrine differentiation (NED). However, treating NEPC is difficult, as no potent drugs are available for this type of cancer progression. We plan to follow up and investigate the effect of *Salmonella* bacteriotherapy on neuroendocrine type tumor cells.

Conclusions

In summary, in this study we showed that weekly injections of 10^5–10^7 of bacteriotherapeutic *Salmonella* strain CRC2631 in the TRAMP mouse prostate tumor progression model during tumor development over weeks 10–22 are well tolerated and increased administration of *Salmonella* results in smaller prostate-associated tumors in the mouse groups that reached study endpoint; however, TRAMP tumor progression was unaffected by administration of CRC2631 *Salmonella* monotherapy. Additionally, we observed multiple instances of neuroendocrine-type tumor tissue at the periurethral region in TRAMP prostate cancer mice, associating the TRAMP prostate cancer model with more aggressive androgen receptor negative neuroendocrine human prostate cancers seen after androgen deprivation therapy in human patients.

Using this baseline data, investigators can now proceed to studies employing *Salmonella*-delivered combination chemotherapies in the immunocompetent, autochthonous, neuroendocrine TRAMP prostate tumor model and look for improved tumor resolutions with different types and concentrations of *Salmonella*-delivered combination chemotherapies.

Supporting Information

S1 Fig. Statistical survival curve analysis. The top graph shows the survival curves for all four groups with 95% confidence intervals. The bottom table is the product-limit survival estimates for the four groups without 95% confidence intervals.
(PDF)

S1 File. Pain/distress evaluation for rodents. TRAMP mice that scored 1.0 or greater on the pain/distress evaluation were euthanized after consultation with the attending veterinarian.
(PDF)

S1 Table. Statistical analysis of survival data between *Salmonella* injection groups. The Type 3 Tests for differences between the four groups gives a Wald Chi-Square of 2.6188 with 3 degrees of freedom (DF) and corresponds with a p-value of 0.4542. This tells us that there is not sufficient evidence to conclude that there are any differences in survival among the four groups. Further, we can look at the Analysis of Maximum Likelihood Estimates to see the Hazard Ratio (compared to the control group) and the p-values for comparison to the control group. All p-values are greater than 0.05 so we conclude that there is not sufficient evidence to conclude that any of the groups (10^5, 10^6, 10^7) have significantly different survival than the control group.
(PDF)

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Author Contributions

Conceived and designed the experiments: RAK.

Performed the experiments: RAK TM.

Analyzed the data: RAK BG TM.

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