Crull, K., Bumann, D., Weiss, S.
Influence of infection route and virulence factors on colonization of solid tumors by Salmonella enterica serovar Typhimurium
(2011) FEMS Immunology and Medical Microbiology, 62 (1), pp. 75-83.

Influence of infection route and virulence factors on colonization of solid tumors by *Salmonella enterica* serovar Typhimurium

Katja Crull[1*], Dirk Bumann[2], Siegfried Weiss[1]

1) Molecular Immunology, Helmholtz Centre for Infection Research, Inhoffenstr. 7, D-38124 Braunschweig, Germany

2) Institute for Microbiology, Biocenter, University Basel, Klingelbergstraße 50/70; Ch-4056 Basel, Switzerland

Running title: Tumor targeting by *S. typhimurium*

Key words: *S. typhimurium*; tumor colonization, virulence factors, mutants,

*corresponding author : Molecular Immunology, HZI – Helmholtz Centre for Infection Research, Inhoffenstr. 7, 38124 Braunschweig, Germany,

Tel.: +49-531-6181 5113, Fax.: +49-531-6181 5002,

E-mail: katja.kochrübe@helmholtz-hzi.de

Abstract

Administration of facultative anaerobic bacteria like *Salmonella enterica* serovar Typhimurium (*S. typhimurium*) as anti-cancer treatment holds a great therapeutic potential. Here we tested different routes of application of *S. typhimurium* with regard to tumor colonization and therapeutic efficacy. No differences between iv and ip infection were observed, often leading to a complete tumor clearance. In contrast, after oral application tumor colonization was inefficient and delayed. No therapeutic effect was observed under such conditions. We also could show that tumor invasion and colonization was independent of functional SPI 1 and SPI 2. Furthermore, tumor invasion and colonization did not require bacterial motility nor chemotactic responsiveness. The distribution of the bacteria within the tumor was independent of such functions.

Introduction

For almost 200 years it is known that bacteria have the ability to colonize solid tumors and induce tumor shrinkage. Despite some success, the employment of bacteria or bacterial components was only anecdotal due to the severe side effects of such therapies (Coley, 1893). However, the dramatic improvements of molecular genetics of bacteria within the last decades now render the application of appropriately attenuated pathogenic bacteria to cancer patients feasible. Consequently, this possibility is presently under intense investigation (Leschner & Weiss, 2010).

Many obligate and facultative anaerobic bacteria are able to colonize solid tumors amongst them *Salmonella enterica* serovar Typhimurium (*S. typhimurium*). Thus far, *S. typhimurium* has been shown to exert strong therapeutic effects on tumors upon intravenous (iv) administration (Zhao et al., 2005). In the tumor, *Salmonella* mainly resides in the inner necrotic part of the neoplasia (Avogadri et al., 2005; Pawelek et al., 1997a; Westphal et al., 2008b). Obviously, conditions like low oxygen tension, protection from phagocytic immune cells and probably also the high nutrient supply from dying tumor cells support survival and proliferation of the bacteria within the tumor. In contrast, a strain , auxotrophic for leucine and arginine, targets tumor tissue specifically, including the complete viable malignant tissue (Hayashi et al., 2009; Kimura et al., 2010; Zhao et al., 2006). Normally, *S. typhimurium* uses an intestinal port of entry via ingestion of contaminated food and water (Jones et al., 1992). After breaching the epithelial barrier the bacteria colonize Peyer´s patches, mesenteric lymph nodes and subsequently spread to the deep organs spleen and liver (Barthel et al., 2003; Finlay & Brumell, 2000). Different components of *Salmonella* are involved in the invasion and infection process. For instance, the cell envelope component lipopolysaccharide is important for survival in the host (Gunn, 2008). In addition, particular virulence factors are found, most of which are encoded in particular genetic elements including Salmonella pathogenicity islands 1 and 2 (SPI 1 and SPI 2). Their prominent features are so-called type three secretion systems (TTSS) that allow the injection of bacterial effector proteins into the cytosol of the host cell (Bueno et al., 2005; Jones et al., 2007; Stecher et al., 2004b). Here, SPI 1 is important for invasion of host epithelial cells (Main-Hester et al., 2008; Waterman & Holden, 2003) while SPI 2 is essential for intracellular survival in the Salmonella containing vacuoles (SCV) after invasion (Cirillo et al., 1998).

For successful *Salmonella*-mediated tumor therapy bacteria are generally administered iv. However, some reports described an application via the natural oral route that successfully inhibited tumor growth (Fest et al., 2009; Medina et al., 1999; Panthel et al., 2008). Nevertheless, a comparison of different requirements of bacterial administration for tumor therapy has only to some extend been approached.

In addition, the tumor invasion process of the systemically applied salmonella is not yet clear although this knowledge will be absolutely required for optimization of the bacterial targeting of cancerous tissue. An active scenario that involves several bacterial chemotactic systems has been suggested using cylindroids of tumor cells *in vitro (Forbes et al., 2003)*. From our studies, we suggest a rather passive mechanism by which the cytokines that are elicited after iv application of the *Salmonella* open the blood vessels in the tumors and allow the entry of the bacteria. Similar results published by the Szalay group also support a passive tumor mechanism (Stritzker et al., 2010). However, both scenarios are not mutual exclusive and need to be investigated in more detail.

In addition, a controversy exists with regard to the involvement of SPI 1 or SPI 2 in tumor targeting and survival. Since the bacteria are normally applied iv, factors encoded in SPI 1 should not be necessary. However, products of SPI 2 were described to be essential for tumor targeting (Pawelek et al., 2002b). This would suggest that bacteria might exist intracellularly in the tumors. Thus far, when examining infected tumors we hardly ever found bacteria residing within cells. Therefore we wanted to re-examine the requirement of intact SPI 1 and SPI 2 for systemic tumor therapy.

Materials and Methods

Bacterial strains and growth conditions

S. typhimurium strain SL7207 (Δ*hisG*, Δ*aroA*) was kindly provided by Bruce Stocker (Hoiseth & Stocker, 1981). For in vivo imaging *lux* was integrated into the chromosome (Loessner et al., 2007b). S. *typhimurium* 1344 and the mutants Δ*cheY*, Δ*fliGHI*, Δ*invG*, Δ*phoP*, Δ*sseD*, Δ*ssrB*, Δ*aroA* and Δ*purA* (Tab. 1) have been provided by Wolf-Dietrich Hardt (Hapfelmeier et al., 2005; Stecher et al., 2004a).The strains have been prepared via deletion of the described genes (Datsenko & Wanner, 2000). The bacteria were grown on LB agar plates supplemented with 30 µg/ml streptomycin at 37°C.

Cell lines and animals

6 week old, female BALB/c mice were purchased from Janvier (France). CT26 colon carcinoma cells (ATCC CRL-2638) were grown as monolayers in IMDM Medium (Gibco BRL, Germany) supplemented with 10% (v/v) heat-inactivated fetal calf serum (Integro, The Netherlands), 250 µmol/l β-Mercaptoethanol (Serva) and 1% (v/v) penicillin/streptomycin (Sigma, Germany).

Infection of tumor bearing mice and recovery of bacteria from tissue

6 week old, female BALB/c mice were subcutaneously injected with $5x10^5$ CT26 cells at the abdomen. When the tumors had reached a size of 5-8 mm in diameter, mice were injected iv or intraperitoneally (ip) with $5x10^6$ CFU of *S. typhimurium* in phosphate-buffered saline (PBS). For oral infection $5x10^8$ CFU were used. At different time points post infection (pi), mice were sacrificed and tumor, spleen and liver were removed for further analysis. The organs were transferred into 1 ml (2 ml for liver) ice-cold PBS containing 0.1 % (v/v) Triton X-100. The tissue was homogenized with a Polytron PT3000 homogenizer. To determine CFU per g of organ, homogenates were serial diluted in PBS and plated on LB plates containing streptomycin (30 µg/ml).

All animal experiments have been performed with the permission of the local authorities (LAVES) according to the animal welfare act.

Tumor studies

To follow the development of tumor size 6 week old female BALB/c mice were injected subcutaneously with 5 x 10^5 CT26 cells. As soon as small tumors become visible, tumor size was monitored every other day using a caliper. The volume of the tumor was calculated using the formula: $V = 4/3 \times \pi \times (h \times w^2) / 8$; h = height and w = width. It was assumed that depth and width of the tumor are equal.

In vivo imaging

For the visualisation of early time points of bacterial spreading after infection the IVIS system (Xenogen) was used. The *lux* operon was used as reporter system for the distribution of bacteria. BALB/c mice were infected iv, ip or orally with *SL7207lux* and immediately anestesized using Isofluran for *in vivo* imaging. Pictures were taken directly, 10min, 2h and 24h after infection with a comparable intensity scale.

Histology

Tumors were taken from sacrificed mice and frozen in Tissue-Tek OCT Compound (Sakura Finetek). Sections of 10 µm were cut using a microtome-cryostat (Cryo-Star HM560V Microm). Slides were air dried at room temperature over night and fixed in -20°C acetone for 3 min. Slides were rehydrated in PBS and blocked with 1 µg / ml FcR blocker (rat-α-mouse CD16/CD32). Staining was done with the following reagents: polyclonal rabbit anti-*S. typhimurium* (USBiological), polyclonal goat anti-rabbit Alexa 488 (Sigma-Aldrich), Phalloidin Alexa 594 (Molecular Probes) and DRAQ5 (Biostatus). Afterwards, the slides were washed and dried and covered with Neomount (Merck) and cover slips. The samples were analyzed using a laser scanning confocal microscope (LSM 510 META, Zeiss) Images were processed with LSM5 Image Browser (Zeiss) and Adobe Photoshop 7.0.

Results

Comparison of application routes for bacterial tumor colonization

The natural port of entry for *S. typhimurium* is the intestine. However, the tumor targeting experiments are usually carried out via the iv route although some reports describe oral application. We therefore wanted to systematically compare the various infection routes for their efficiency with respect to tumor colonization. Within these experiments we employed SL1344Δ*aroA*. This strain is auxotrophic for histidine (Wray & Sojka, 1978) as well as aromatic amino acids. Mice were injected subcutaneously with the colon carcinoma cell line CT26. After the tumor had reached a size of 5mm in diameter, the tumor bearing mice were infected by different routes with the attenuated *S. typhimurium* strain. After iv and ip infection, mice were sacrificed on day 1, 3, 10 and 17 after infection and the number of bacteria was determined for tumor, spleen and liver (Fig. 1). As expected, tumors were preferentially colonized and show up to 100 times higher colonization than spleen and liver. Bacterial tumor loads decreased slightly till day 17. In contrast, in normal target organs spleen and liver bacteria increased till day 3 and then slowly subsided.
Due to delayed tissue colonization in case of oral infection, mice were sacrificed at day 2, 6, 11, 15 and 22 post infection and tissues were plated. Bacteria could only be detected at day 11. The tumors were preferentially colonized but to a much lower degree than after systemic application. In general, the number of bacteria in the tissues was about 100 times lower compared to iv or ip infection.

Under such conditions, also development of tumor size was monitored. After iv and ip infection, a dramatic reduction of tumor volume was observed (Fig. 2). The tumor colonization of SL1344Δ*aroA* was reflected in a complete clearance of tumors. Consistently, after oral infection, which had led to a transient low colonization of the tumor, no reduction of tumor size was detectable.

The colonization of organs and tumor was also followed via *in vivo* imaging (Fig.3). Recombinant bacteria of the strain SL7207*lux,* carrying an integrated *lux* operon, were used to visualize bacterial distribution during early infection. Bacteria of the strain SL7207 contain the same attenuations and were shown to behave comparable to SL1344Δ*aroA.* The amount of Cfu was lower in different organs but the pattern of infection and survival of mice was comparable. (Supl. Fig. 1 and 2). After iv infection bacteria immediately spread out in the whole organism. Already after 10 minutes a strong signal from liver and spleen is detectable. After 24h an additional very strong signal from the tumor appears. After ip infection the signal

from liver and spleen is not that obvious due to long-lasting strong signal from the peritoneum. Nevertheless, after 24h the tumor shows strong bioluminescence.

In contrast, only a 100 times weaker signal is observed directly after oral infection. Bacteria can be detected in the gut up to a few hours after infection. After 24h the signal is too weak for detection.

Tumor colonization by metabolically attenuated mutants

The employment of SL1344 allowed the comparison of WT bacteria with mutant bacteria (Table 1) that were mutated in metabolic pathways, commonly used for attenuation of vaccine carrier strains (Fig. 4). Therefore, we compared WT bacteria with Δ*aroA* and Δ*purA*, which are unable to synthesize either aromatic amino acids or purines like adenine and guanine. Interestingly, at 12 h post infection wild type bacteria were able to colonize tumors as well as spleen in a higher degree than the attenuated strains. By 24 h, bacteria of the Δ*aroA* strain had colonized tumor and spleen to the same extend as the WT. The Δ*purA* strain however, never grew up to such numbers and appears to be over attenuated. After 2 days all mice had to be killed due to a severe health status after iv infection with WT SL1344 while most of the mice infected with the mutant strains recovered from side effects of the infection.

Tumor colonization by mutants in SPI 1 and SPI 2

The pathogenicity islands SPI 1 and 2 are essential for bacterial invasion and intracellular survival under natural conditions, respectively. However, in the case of tumor targeting such functions might be dispensable since the Salmonella are applied intravenously and appear to reside extracellularly in the tumor tissue (Loessner et al., 2007a). We therefore tested the bacterial mutants Δ*invG* and Δ*phoP,* that are essential for the function of SPI 1, whereby Δ*phoP* is also influencing SPI 2, or Δ*sseD* and Δ*ssrB* mutants*,* that abolish the function of SPI 2, for tumor invasion and colonization. Efficiency of tumor invasion and survival was obviously not affected by such mutations (Fig. 4). The mutants invaded the tumor even slightly better than the wild type bacteria as seen at 12 h pi. By 24 h, no difference between bacterial counts was observable anymore. Thus, virulence factors of *S. typhimuirum* encoded in SPI 1 and 2 are not essential for tumor colonization under our conditions.

Tumor colonization by immobile variants and variants with chemotaxis defects

Conflicting ideas exist as to how the bacteria enter the tumor tissue. An active process was suggested that required motility and chemotaxis by the bacteria (Kasinskas & Forbes, 2006; Kasinskas & Forbes, 2007a). These experiments were carried out *in vitro* using tumor cell cylindroids. Hence, we wanted to test the requirement of motility and chemotaxis under our conditions *in vivo.* Two variant strains were tested: mutant Δ*fliGHI* is immobile due to an inability to assemble flagella and Δ*cheY* is unable to respond to chemotactic gradients since it can no longer regulate the rotation of the flagella.

Importantly, both mutant strains invaded the tumors slightly better than the WT bacteria, as can be seen at 12 h pi. No difference could be observed at 24 h anymore indicating that tumor invasion and colonization is independent of bacterial motility and chemotaxis. No difference between such strains could be observed for the colonization of the spleen at both time points.

Tumor distribution of non-motile variant bacteria

Under our conditions, tumor colonizing Salmonella reside in the necrotic areas and in the areas between the necrotic and the viable region, in which normally also high numbers of host neutrophils are found (Rosenberg et al., 2002; Westphal et al., 2008a). It is possible that the deletion of the motile or chemotactic capabilities of the variant strains might influence their distribution in the tumor. Therefore, tumors colonized by WT, Δ*cheY* or Δ*fliGHI* bacteria were analyzed by immunohistology 24 h pi. As can be seen from Fig. 5, bacteria of the different strains were similarly distributed within the tumor. All of them colonized the necrotic areas and accumulated to some extend in the interface between necrotic and viable regions. Thus, motility and chemotaxis appears not to be required for tumor invasion and colonization under our conditions.

Discussion

In industrialized countries cancer is the second most frequent cause of death with an increasing incidence. This is due to the improved live expectancy of the human population as cancer is essentially a disease of old age. In face of these facts, the development of novel therapies or intervention strategies against this devastating disease is an obligatory task for present biomedical research. Despite of the draw backs of the first clinical trials (Toso et al., 2002), the employment of bacteria for the treatment of solid tumors holds great promises. However, it also became clear that the understanding of the invasion and colonization process is essential for a further development of efficacious strains that can be safely administered to tumor patients. Here, we have investigated the role of some bacterial properties in tumor invasion and colonization, for which conflicting ideas existed.

Tumor targeting bacteria are usually applied systemically. However, a few reports describe the successful application via the oral route. Obviously, an oral delivery of therapeutic bacteria would be advantageous since it would not require any special equipment nor any particular skills for delivery. In addition, the risk of an overreaction of the immune system of the patient would be reduced. However, comparison of the different routes of application revealed that under our conditions systemic administration of the bacteria is much superior to the oral route. Oral application of SL1344*ΔaroA* only led to a transient colonization of the tumors late after infection. In contrast, systemic application of bacteria led to an immediate and robust colonization of the tumor. In accordance, tumor growth was unaltered when the bacteria were applied orally while systemic application led to a strong shrinkage of the tumors and resulted in complete clearance. Thus, *Salmonella* when applied orally are able to reach the tumor tissue but their present therapeutic capabilities render the effects negligible. Nevertheless, appropriately engineered strains might eventually allow oral application for therapeutic purposes.

During these experiments we noticed that the therapeutic effect was very much dependent on the size of the tumor. Our standard conditions to apply the bacteria when the tumor has reached 0.5 – 0.8 cm in diameter allows complete tumor clearance by the systemic applied *Salmonella*. In contrast, less therapeutic efficacy is noticed when the tumor had reached larger sizes. This might be one of the reasons why the bacteria after oral application at 0.5 cm in diameter did not exhibit any effect on tumor growth. Despite the lower number of colonizing bacteria a retardation of tumor growth was expected. However, when the orally applied bacteria had reached the tumor it might have been beyond the critical size for a therapeutic effect.

Since the mutant strains employed during these studies were generated on the SL1344 genetic background, but for *in vivo* imaging of early time points of infection SL7207*lux* was used. SL1344Δ*aroA* and SL7207 were compared regarding their colonization pattern and therapeutic effect on tumor-bearing mice (Sup.Fig 1 and 2). Only slight differences could be observed. SL7207 colonized various target tissues less efficient than SL1344Δ*aroA*. Both strains also differ slightly in their capacity to induce tumor shrinkage. While infection with SL1344Δ*aroA* induced complete tumor clearance, treatment with SL7207 ended only in the elimination of 3 tumors out of 5. This might be due to differences in the genetic make up of the two strains. Nevertheless, the numbers of bacteria during colonization and especially the kinetics are comparable.

Metabolic attenuation in the purine synthesis pathway, like Δ*purI*, is often used in studies concerning tumor targeting of Salmonella (Clairmont et al., 2000; Pawelek et al., 1997b). Such strains usually exhibit good performance. In contrast, the SL1344Δ*purA,* used in our study, appeared over attenuated.

Under our conditions, we found that Salmonella usually residing extracellularly after systemic application. This suggested that proteins encoded in the pathogenicity islands SPI 1 and SPI 2 should not be essential for tumor invasion and colonization. This was indeed the case. Mutants, in which essential functions of SPI 1 or SPI 2 were deleted, were efficient tumor colonizers. Thus, the original description that SPI 2 functions are required for tumor colonization (Pawelek et al., 2002a) could not be confirmed. Instead of our CT26 colon carcinoma model those experiments were carried out in a melanoma model, in which the bacteria might behave in another way. Also different strain backgrounds were used and might explain the different outcome of experiments.

Similarly, an active process was suggested from *in vitro* data for tumor invasion (Kasinskas & Forbes, 2007b). This would require motility and chemotactic responsiveness of the bacteria. However, in our system variant *Salmonella* that do not have such properties invaded and colonized the tumors just as efficient as bacteria exhibiting these properties. Therefore, we feel that our original hypothesis is correct, namely that *Salmonella* after systemic application via induction of TNF-α and other cytokines creates a tumor microenvironment, where the bacteria are passively flushed in (Leschner et al., 2009). Even the distribution of the *Salmonella* within the tumor is independent of motility and chemotactic responsiveness. Obviously, the test of more and independent tumor systems will be required to be able to generalize these findings.

Taken together, we have tested several properties of tumor targeting bacteria. Our results will help to better understand the tumor invasion and colonization process for this novel and highly potential therapeutic strategy.

Acknowledgement

This work was supported in part by the the Bundesministerium für Bildung und Forschung (BMBF), Deutsche Forschungsgemeinschaft (DFG), the Deutsche Krebshilfe and the Helmholtz Graduate School for Infection Research.

We want to thank Susanne zur Lage, Regina Lesch and Martina Krey for expert technical assistance.

311 Reference List

312

313 Avogadri F, Martinoli C, Petrovska L, Chiodoni C, Transidico P, Bronte V, Longhi R,
314 Colombo MP, Dougan G, & Rescigno M. (2005) Cancer immunotherapy based
315 on killing of Salmonella-infected tumor cells. *Cancer Res* **65:** 3920-3927.

316 Barthel M, Hapfelmeier S, Quintanilla-Martinez L, Kremer M, Rohde M, Hogardt M,
317 Pfeffer K, Russmann H, & Hardt WD. (2003) Pretreatment of mice with
318 streptomycin provides a Salmonella enterica serovar Typhimurium colitis
319 model that allows analysis of both pathogen and host. *Infect Immun* **71:** 2839-
320 2858.

321 Bueno SM, Tobar JA, Iruretagoyena MI, & Kalergis AM. (2005) Molecular interactions
322 between dendritic cells and Salmonella: escape from adaptive immunity and
323 implications on pathogenesis. *Crit Rev Immunol* **25:** 389-403.

324 Cirillo DM, Valdivia RH, Monack DM, & Falkow S. (1998) Macrophage-dependent
325 induction of the Salmonella pathogenicity island 2 type III secretion system
326 and its role in intracellular survival. *Mol Microbiol* **30:** 175-188.

327 Clairmont C, Lee KC, Pike J, Ittensohn M, Low KB, Pawelek J, Bermudes D, Brecher
328 SM, Margitich D, Turnier J, Li Z, Luo X, King I, & Zheng LM. (2000)
329 Biodistribution and genetic stability of the novel antitumor agent VNP20009, a
330 genetically modified strain of Salmonella typhimurium. *J Infect Dis* **181:** 1996-
331 2002.

332 Coley WB. (1893) The treatment of malignant tumors by repeated inoculations of
333 erysipelas. With a report of ten original cases. *Clin Orthop Relat Res* **262:** 3-
334 11.

335 Datsenko KA & Wanner BL. (2000) One-step inactivation of chromosomal genes in
336 Escherichia coli K-12 using PCR products. *Proc Natl Acad Sci U S A* **97:**
337 6640-6645.

338 Fest S, Huebener N, Bleeke M, Durmus T, Stermann A, Woehler A, Baykan B,
339 Zenclussen AC, Michalsky E, Jaeger IS, Preissner R, Hohn O, Weixler S,

340 Gaedicke G, & Lode HN. (2009) Survivin minigene DNA vaccination is
341 effective against neuroblastoma. *Int J Cancer* **125:** 104-114.

342 Finlay BB & Brumell JH. (2000) Salmonella interactions with host cells: in vitro to in
343 vivo. *Philos Trans R Soc Lond B Biol Sci* **355:** 623-631.

344 Forbes NS, Munn LL, Fukumura D, & Jain RK. (2003) Sparse initial entrapment of
345 systemically injected Salmonella typhimurium leads to heterogeneous
346 accumulation within tumors. *Cancer Res* **63:** 5188-5193.

347 Gunn JS. (2008) The Salmonella PmrAB regulon: lipopolysaccharide modifications,
348 antimicrobial peptide resistance and more. *Trends Microbiol* **16:** 284-290.

349 Hapfelmeier S, Stecher B, Barthel M, Kremer M, Muller AJ, Heikenwalder M,
350 Stallmach T, Hensel M, Pfeffer K, Akira S, & Hardt WD. (2005) The
351 Salmonella pathogenicity island (SPI)-2 and SPI-1 type III secretion systems
352 allow Salmonella serovar typhimurium to trigger colitis via MyD88-dependent
353 and MyD88-independent mechanisms. *J Immunol* **174:** 1675-1685.

354 Hayashi K, Zhao M, Yamauchi K, Yamamoto N, Tsuchiya H, Tomita K, & Hoffman
355 RM. (2009) Cancer metastasis directly eradicated by targeted therapy with a
356 modified Salmonella typhimurium. *J Cell Biochem* **106:** 992-998.

357 Hoiseth SK & Stocker BA. (1981) Aromatic-dependent Salmonella typhimurium are
358 non-virulent and effective as live vaccines. *Nature* **291:** 238-239.

359 Jones BD, Lee CA, & Falkow S. (1992) Invasion by Salmonella typhimurium is
360 affected by the direction of flagellar rotation. *Infect Immun* **60:** 2475-2480.

361 Jones MA, Hulme SD, Barrow PA, & Wigley P. (2007) The Salmonella pathogenicity
362 island 1 and Salmonella pathogenicity island 2 type III secretion systems play
363 a major role in pathogenesis of systemic disease and gastrointestinal tract
364 colonization of Salmonella enterica serovar Typhimurium in the chicken. *Avian*
365 *Pathol* **36:** 199-203.

366 Kasinskas RW & Forbes NS. (2006) Salmonella typhimurium specifically chemotax
367 and proliferate in heterogeneous tumor tissue in vitro. *Biotechnol Bioeng* **94:**
368 710-721.

Kasinskas RW & Forbes NS. (2007b) Salmonella typhimurium lacking ribose chemoreceptors localize in tumor quiescence and induce apoptosis. *Cancer Res* **67:** 3201-3209.

Kasinskas RW & Forbes NS. (2007a) Salmonella typhimurium lacking ribose chemoreceptors localize in tumor quiescence and induce apoptosis. *Cancer Res* **67:** 3201-3209.

Kimura H, Zhang L, Zhao M, Hayashi K, Tsuchiya H, Tomita K, Bouvet M, Wessels J, & Hoffman RM. (2010) Targeted therapy of spinal cord glioma with a genetically modified Salmonella typhimurium. *Cell Prolif* **43:** 41-48.

Leschner S & Weiss S. (2010) Salmonella-allies in the fight against cancer. *J Mol Med .* **88:** 763-673

Leschner S, Westphal K, Dietrich N, Viegas N, Jablonska J, Lyszkiewicz M, Lienenklaus S, Falk W, Gekara N, Loessner H, & Weiss S. (2009) Tumor invasion of Salmonella enterica serovar Typhimurium is accompanied by strong hemorrhage promoted by TNF-alpha. *PLoS One* **4:** 6692.

Loessner H, Endmann A, Leschner S, Westphal K, Rohde M, Miloud T, Hammerling G, Neuhaus K, & Weiss S. (2007a) Remote control of tumour-targeted Salmonella enterica serovar Typhimurium by the use of L-arabinose as inducer of bacterial gene expression in vivo. *Cell Microbiol* **9:** 1529-1537.

Main-Hester KL, Colpitts KM, Thomas GA, Fang FC, & Libby SJ. (2008) Coordinate regulation of Salmonella pathogenicity island 1 (SPI1) and SPI4 in Salmonella enterica serovar Typhimurium. *Infect Immun* **76:** 1024-1035.

Medina E, Guzman CA, Staendner LH, Colombo MP, & Paglia P. (1999) Salmonella vaccine carrier strains: effective delivery system to trigger anti-tumor immunity by oral route. *Eur J Immunol* **29:** 693-699.

Panthel K, Meinel KM, Sevil D, V, Trulzsch K, & Russmann H. (2008) Salmonella type III-mediated heterologous antigen delivery: a versatile oral vaccination strategy to induce cellular immunity against infectious agents and tumors. *Int J Med Microbiol* **298:** 99-103.

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

Pawelek JM, Sodi S, Chakraborty AK, Platt JT, Miller S, Holden DW, Hensel M, & Low KB. (2002a) Salmonella pathogenicity island-2 and anticancer activity in mice. *Cancer Gene Ther* **9:** 813-818.

Rosenberg SA, Spiess PJ, & Kleiner DE. (2002) Antitumor effects in mice of the intravenous injection of attenuated Salmonella typhimurium. *J Immunother* **25:** 218-225.

Stecher B, Hapfelmeier S, Muller C, Kremer M, Stallmach T, & Hardt WD. (2004a) Flagella and chemotaxis are required for efficient induction of Salmonella enterica serovar Typhimurium colitis in streptomycin-pretreated mice. *Infect Immun* **72:** 4138-4150.

Stritzker J, Weibel S, Seubert C, Gotz A, Tresch A, van Rooijen N, Oelschlaeger TA, Hill PJ, Gentschev I, & Szalay AA. (2010) Enterobacterial tumor colonization in mice depends on bacterial metabolism and macrophages but is independent of chemotaxis and motility. *Int J Med Microbiol*

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, & Rosenberg SA. (2002) Phase I study of the intravenous administration of attenuated Salmonella typhimurium to patients with metastatic melanoma. *J Clin Oncol* **20:** 142-152.

Waterman SR & Holden DW. (2003) Functions and effectors of the Salmonella pathogenicity island 2 type III secretion system. *Cell Microbiol* **5:** 501-511.

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

Wray C & Sojka WJ. (1978) Experimental Salmonella typhimurium infection in calves. *Res Vet Sci* **25:** 139-143.

Zhao M, Yang M, Li XM, Jiang P, Baranov E, Li S, Xu M, Penman S, & Hoffman RM. (2005) Tumor-targeting bacterial therapy with amino acid auxotrophs of GFP-expressing Salmonella typhimurium. *Proc Natl Acad Sci U S A* **102:** 755-760.

Zhao M, Yang M, Ma H, Li X, Tan X, Li S, Yang Z, & Hoffman RM. (2006) Targeted therapy with a Salmonella typhimurium leucine-arginine auxotroph cures orthotopic human breast tumors in nude mice. *Cancer Res* **66:** 7647-7652.

435 **Table 1.** Mutant strains of *S. typhimurium*

SL1344	Affecting	Function
Δ*cheY*	Chemotaxis	Regulator of flagella rotation; mutants are unable to respond to chemotactic signals
Δ*fliGHI*	Flagella	Assembly of flagella structural proteins
Δ*invG*	SPI 1	Component of SPI 1 TTSS response regulator of two-component transcriptional regulator
Δ*phoP*	SPI 1/2	phoP/phoQ
Δ*sseD*	SPI 2	Translocon protein of SPI 2 TTSS; part of two-component regulatory system ssrA/ ssrB pf SPI 2
Δ*ssrB*	SPI 2	TTSS
Δ*aroA*	Metabolism	Biosynthesis of aromatic aminoacids
Δ*purA*	Metabolism	Biosynthesis of adenine and guanine

436

437 **Legends**

438 **Fig. 1.** Colonization of tumor, spleen and liver after iv, ip and oral application of SL1344
439 Δ*aroA*. Bars show the mean of 5 mice per group ± SDM.

440

441 **Fig. 2.** Therapeutic effect of the colonization of tumor-bearing mice by SL1344Δ*aroA*. The
442 infection was carried out 9 days after the administration of tumor cells and the development
443 is given as percent of tumor size at day 5. The values shown here are mean values of 5 mice
444 per group ± SDM.

445

446 **Fig. 3.** Colonization of tumor-bearing mice infected with SL7207*lux*, carrying chromosomally
447 integrated *lux*, after iv, ip or oral application. Note that the scale is different for iv / ip and oral
448 infection. Position of tumors are indicated by black arrows.

449 **Fig. 4.** Colonization of tumor-bearing mice by mutants of SL1344 12 and 24 h after iv
450 infection. Tumor and spleen were tested for the number of bacteria per g of tissue. Bars

show mean value of 5 mice per group ± SDM. Statistical significance, calculated via Student´s t-test, is indicated by asterisks; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.005$.

Fig. 5. Immune histology of tumors colonized by WT SL1344, immobile mutants (Δ*fliGHI*) or a mutant strain, unable to react to chemotactic gradients (Δ*cheY*). 10 µm cryo sections were stained for nuclei (blue), actin (red) and *S. typhimurium* (green). Confocal images show overviews (10x) and 40x magnifications of the area in boxes.

Supl. Fig. 1. Comparison of the colonization of tumor, spleen and liver after iv, ip and oral application. Cfu per g tissue were compared after infection with SL7207 and SL1344Δ*aroA.*

Supl. Fig. 2. Comparison of the therapeutic effect of SL7207 and SL1344Δ*aroA* to tumor-bearing mice. The infection was carried out 9 days after the administration of tumor cells and the development is given as percent of tumor size at day 5.