Loessner, H., Endmann, A., Leschner, S., Westphal, K., Rohde, M., Miloud, T., Ha?mmerling, G., Neuhaus, K., Weiss, S.
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(2007) Cellular Microbiology, 9 (6), pp. 1529-1537.

Remote control of tumor targeted *Salmonella enterica* serovar Typhimurium by the use of L-arabinose as inducer of bacterial gene expression *in vivo*

running title : Remote control of tumor targeted *Salmonella*

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keywords : Salmonella; remote controlled gene expression; bacterial vector; tumor gene therapy, P_{BAD}, L-arabinose

Abstract

We have used *Salmonella enterica* serovar Typhimurium (*S. typhimurium*) which are able to colonize tumors besides spleen and liver. Bacteria were equipped with constructs encoding GFP or luciferase as reporters under control of the promoter P_{BAD} that is inducible with L-arabinose. Reporter genes could be induced in culture but also when the bacteria resided within the mouse macrophages J774A.1. More important, strong expression of reporters by the bacteria could be detected in mice after administration of L-arabinose. This was especially pronounced in bacteria colonizing tumors. Histology demonstrated that the bacteria had accumulated in and close to necrotic areas of tumors. Bacterial gene induction was observed in both regions. P_{BAD} is tightly controlled also *in vivo* since gene E of bacteriophage ΦX174 could be introduced as inducible suicide gene. The possibility to deliberately induce genes in bacterial carriers within the host should render them extremely powerful tools for tumor therapy.

Introduction

Bacteria and bacterial products have a long history as tumor therapeutics (Pawelek *et al.*, 2003). Recently, several strictly or facultative anaerobic bacteria have attracted particular attention because they display the ability to target and accumulate in solid tumors. Such bacteria were adapted to deliver therapeutic factors and were applied alone or in combination with conventional therapeutics (Dang *et al.*, 2001; Jain and Forbes, 2001; Theys *et al.*, 2003).

S. typhimurium, a motile, invasive Gram-negative bacterium, is able to colonize solid tumors. Chemoattractive compounds produced by quiescent tumor cells have been shown to contribute to this effect *in vitro* (Kasinskas and Forbes, 2006). *In vivo*, *S. typhimurium* primarily colonizes hypoxic, nutrient rich, necrotic areas (Forbes *et al.*, 2003) but is also able to invade tumor cells (Avogadri *et al.*, 2005; Pawelek *et al.*, 2002). Colonization results in retardation of tumor growth thus prolonging survival of the mice (Clairmont *et al.*, 2000; Low *et al.*, 1999; Pawelek *et al.*, 1997; Zhao *et al.*, 2006). In clinical trials, systemically administered bacteria colonized human tumors at high frequency without causing adverse toxic effects (Nemunaitis *et al.*, 2003).

S. typhimurium has been successfully used to deliver therapeutic molecules to tumors such as prodrug-converting enzymes and antigens (King *et al.*, 2002; Nishikawa *et al.*, 2006; Pawelek *et al.*, 1997). Expression of these molecules was driven by constitutive promoters. However, for the purpose of tumor therapy, inducible systems would be more appropriate. A range of promoters have been characterized that respond to various stimuli, e.g. particular substrates, temperature, pH, ionic strength, hypoxia, radiation or other environmental conditions (Cases and de Lorenzo, 1998). Most of such systems are excluded from *in vivo* use. We concentrated therefore on the P_{BAD} promoter from the arabinose operon of *Escherichia coli*. This system can be activated by the sugar L-arabinose. The P_{BAD}

promoter is widely used to positively control expression in bacterial cultures (Guzman *et al.*, 1995). Here we show that heterologous genes under control of P_{BAD} can also be induced *in vivo* when L-arabinose is administered systemically*. S. typhimurium,* particularly when colonizing tumors, were highly susceptible to remote control*.* This opens a great number of possibilities for improvement of the tumor-specific delivery of therapeutic molecules by bacteria.

Results

L-arabinose induced reporter gene expression in bacterial culture and host macrophages

To investigate the possibility of inducing bacterial gene expression under remote control in the mammalian host, we first established sensitive reporter systems. We constructed plasmids encoding green fluorescent protein (GFP) (Cormack *et al.*, 1996) or firefly luciferase (Fluc) under control of the P_{BAD} promoter. SL7207, an attenuated experimental vaccine strain of *S. typhimurium* (Hoiseth and Stocker, 1981), was transformed with these vectors. Strong induction of GFP or Fluc expression could be observed when L-arabinose was added to cultures of such transformants (Fig. 1A,B). Reporter gene expression ceased after 2 hrs (Fig. 1B), most likely because the bacteria had consumed all the sugar at this time point (Supplementary Fig. 1A). Of note, induction of reporter genes retarded bacterial growth and in parallel bacteria appeared enlarged under such conditions (Supplementary Fig. 1B and C, and data not shown). We then wanted to see whether induction is also possible when the *Salmonella* transformants were residing inside host cells. Therefore, GFP and Fluc transformants were used to infect J774A.1 mouse macrophages. Extracellular bacteria were killed with gentamycin and subsequently L-arabinose was added to the cultures at different concentrations. After incubation for 3 hrs, cells were lysed and bacteria were recovered from the lysate and analyzed for GFP or Fluc expression. As shown in Figure 1C and D, increasing concentrations of L-arabinose in such cultures resulted in increased expression of GFP or Fluc by the bacteria. Furthermore, the number of GFP expressing *Salmonella* increased with rising concentrations of L-arabinose (data not shown).

L-arabinose inducible expression of luciferase in tumor targeted S. typhimurium

Before testing the induction of gene expression in tumor targeted *S. typhimurium in vivo,* we first determined whether sufficient L-arabinose can accumulate in the circulation of mice to induce the P_{BAD} promoter. Figure 2A shows that after intraperitoneal administration, a high but transient concentration of the sugar could be found in the blood of mice. The decrease after 4-6 hrs is probably due to degradation by host enzymes (Seri *et al.*, 1996).

S. typhimurium SL7207 carrying the Fluc reporter plasmid was therefore injected intravenously into mice bearing a subcutaneous CT26 tumor of roughly 0.5 cm in diameter. Three days later, L-arabinose was injected intraperitoneally and colonization as well as Fluc expression by bacteria from tumor, spleen and liver was determined. As can be seen from Figure 2B, more than 10-fold enrichment of bacteria in tumors was observed. This was not as dramatic as previously observed with other *S. typhimurium* strains (Bermudes *et al.*, 2002). We also noticed during all of these experiments that accumulation of SL7207 in tumors was rather variable despite all tumors were colonized (Fig. 2B). Nevertheless, the low accumulation factor and the variability should not influence the proof of principle of remote control of bacterial gene expression *in vivo*.

When Fluc activity was determined in the three tissues, it became clear that by using the P_{BAD} promoter, it is possible to deliberately induce bacterial genes after colonization of the mammalian host. Administration of L-arabinose resulted in a strong induction of Fluc expression in bacteria from all three tissues (Fig. 2C). Interestingly, the induction factor was higher in the tumor than in spleen and liver. This is even more obvious when Fluc activity per CFU was compared (Fig. 2D). Probably, the bacteria are more accessible to the sugar in the tumor or the physiological state of tumor colonizing bacteria results in higher gene expression. Induction of reporter gene expression was specific since administration of equal

amounts of D-glucose or L-rhamnose did not result in detectable Fluc activity when bacteria from tumors and spleen were tested (data not shown). Similarly, a construct where the Fluc gene was under the control of the T7 promoter could not be induced by L-arabinose administration in tumor or spleen colonizing bacteria (data not shown).

Non-invasive in vivo imaging of L-arabinose induced luciferase expression

The bioluminescence that is inducibly generated in bacteria containing Fluc, should be detectable by non-invasive *in vivo* imaging. We therefore injected into tumor bearing mice, SL7207 bacteria that carried plasmids encoding Fluc under the control of P_{BAD} as before. Induction of Fluc was monitored using the supercooled CCD camera system IVIS-100 (Xenogen) after intraperitoneal injection of L-arabinose and luciferin-substrate. As shown in Figure 3A, little Fluc activity is detectable before induction. However, after application of L-arabinose strong Fluc activity becomes readily visible. In concordance with the biochemical assays (Fig. 2C), mainly tumors displayed Fluc activity.

To simplify the non-invasive *in vivo* imaging, we constructed a new *S. typhimurium* strain that contained the *lux* operon of the bacterium *Photorhabdus luminescens* under the control of P_{BAD} as a single copy in the bacterial chromosome of SL7207. No substrate needs to be administered for *lux*. When tumor bearing mice were infected with this bacterial strain and L-arabinose was subsequently administered intraperitoneally, tumor-specific bioluminescence could be detected (Fig. 3C). No light was observed without induction (Fig. 3C,D). With this vector, the intensity of bioluminescence was lower compared to Fluc but the maximal level of light emission was reached earlier (Fig. 3B,D). The lower level of light emission is most likely due to the single copy of the *lux* operon present in the carrier bacteria and a slightly different

emission wavelength of light generated by *lux*. Tumor colonization properties of *lux* bacteria were similar to the strain carrying the *fluc* plasmid (data not shown).

Accessibility of tumor colonizing bacteria to the inducer L-arabinose

Tumor colonizing *S. typhimurium* are supposed to be found in necrotic and hypoxic areas (Forbes *et al.*, 2003; Pawelek *et al.*, 1997). Whether this was also true for strain SL7207 was tested by immune histology. As can be seen in Figure 4A-C, bacteria could be found mainly in necrotic areas and to some extend in bordering areas that are most likely hypoxic. To see whether L-arabinose can reach all bacteria i.e. bacteria in necrotic areas as well as bacteria at the rim, we employed bacteria that carried a low copy number plasmid encoding GFP under the control of P_{BAD}. These bacteria were injected into tumor bearing mice and after administration of L-arabinose, tumor colonizing bacteria were examined for GFP expression by histology. Figures 4D-F show that bacteria expressing GFP can be detected in both regions of the tumor. Thus, L-arabinose probably perfuses all areas of the tumor leading to local concentration that is sufficient to induce the P_{BAD} promoter. No GFP expression could be detected in any region of the tumor when inducer was omitted (data not shown).

In necrotic areas, bacteria are most likely extracellular. This was confirmed by histology (Fig. 4G). However, it was unclear whether in the rim areas SL7207 would invade or reside outside tumor cells. As shown in Figure 4H, in such rim areas most bacteria appear to reside in extracellular spaces and only a few seem to be associated with cells. Transmission electron microscopy confirmed the extracellular location of such bacteria (data not shown).

Whether the bacteria are specifically attracted to these parts of the tumor or whether the presence of the bacteria causes cell death as a consequence of necrosis or

apoptosis, as indicated by the small nuclei, could not be decided (Fig. 4H). Most likely the latter is true since after bacterial colonization the necrotic areas become larger compared to uninfected tumors (data not shown). Nevertheless, tumor growth was not significantly retarded. Cells in non-colonized and thus unaffected regions apparently continued to grow unaltered (Fig. 4I).

Not all bacteria displayed in Figure 4D-F expressed GFP despite retention of the expression plasmid. One explanation for the lack of GFP expression in some bacteria could be the phenomenon of an "all-or-nothing" induction of the P_{BAD} promoter. Subsaturating concentration of L-arabinose inside the tumor could result in gene activation in one but not the other bacterium (Siegele and Hu, 1997). Alternatively, the physiological state of some bacteria might prevent the induction of the reporter gene via P_{BAD}. In concordance with this explanation, when we used bacteria constitutively expressing GFP we also found non-fluorescent bacteria despite retention of the expression plasmid in all bacteria.

L-arabinose inducible bacterial lysis in vitro and in vivo

As a test for tight control *in vivo*, we placed the lysin gene E of the bacteriophage ΦX174 under control of P_{BAD}. Already 100 molecules of this protein should lead to loss of bacterial viability (Maratea *et al.*, 1985). When the construct was integrated as a single copy into the chromosome of the SL7207 bacteria, no interference with growth or integrity in bacterial cultures was observed in the absence of L-arabinose (Fig. 5A,B). However, upon addition of the inducer lysis occurred within 90 min (Fig. 5A) and only bacterial debris could be observed at this time (Fig. 5C).

Whether the same was true *in vivo* was tested with tumor colonizing bacteria. Figure 5D shows that the bacteria preferentially colonized tumors similar to parental bacteria. Administration of L-arabinose resulted in preferential death of bacteria in the

tumor as compared to spleen and liver. This is in concordance with the preferential induction of Fluc in tumor colonizing bacteria described before (Fig. 2C,D). Similarly, only one third of the bacteria were lysed under these circumstances. Thus, the P_{BAD} promoter system is suitable for the introduction of inducible suicide genes into carrier bacteria, which adds important safety features to tumor targeting bacteria.

Discussion

Remote control of gene expression is a new strategy for *in vivo* use of tumor targeting bacteria. To our knowledge, only one bacterial system has been described so far for deliberate induction of genes within the host. It is based on the bacterial response to ionizing irradiation (Nuyts *et al.*, 2001; Theys *et al.*, 2003). However, irradiation itself results in damage of normal tissue and is undermined by low compliance from patients. Thus, use of a low molecular weight biocompatible compound like L-arabinose in combination with the corresponding molecular switch - P_{BAD} - as described in the present work, appears to be ideal for such purpose. P_{BAD} is derived from the *E. coli* arabinose operon and should be transferable to other bacteria.

L-arabinose appears to fulfill all criteria of a versatile inducer. It is a biocompatible food component and suggested for clinical use in other contexts. It not only reaches bacteria that reside in the phagocytic vacuole as shown by our *in vitro* experiments but can also reach bacteria that reside in niches that are provided by tumors. Remote gene induction was indiscriminate of the areas of the tumor. After systemic administration of L-arabinose the sugar only transiently circulates in the blood of mice and is degraded by host enzymes (Seri *et al.*, 1996). Accordingly, expression of genes under control of P_{BAD} is short-lived.

Bioluminescence *in vivo* imaging of light emitting tumor targeted bacteria is an attractive approach in the diagnosis of tumors (Yu *et al.*, 2004). Our approach of transient, L-arabinose mediated induction of bioluminescence emission by the bacteria is sufficient to localize the bacteria inside the tumor but liberates the bacteria from the burden of constant expression of the luciferase.

Only a portion of the bacteria within the same location was induced. The physiological state of bacteria may account for this observation. However, all-or-

nothing induction at sub-saturating inducer concentrations is an alternative explanation. It can be attributed to fluctuating expression of the L-arabinose transporter in an uninduced state of the endogenous arabinose operon (Siegele and Hu, 1997). This might add to the versatility of the system, since induction concerns only a portion of the bacteria and might provide lytic bacteria for repeated release of therapeutic molecules. On the other hand, strains could be engineered that are able to constitutively take up L-arabinose giving rise to homogenous *in vivo* induction (Morgan-Kiss *et al.*, 2002).

The regulation of P_{BAD} is tightly controlled also *in vivo*. A suicide gene could be integrated into the carrier bacteria without interference with growth, invasiveness and tumor colonization. Together with homogenous *in vivo* induction this would introduce an important safety feature into tumor targeting bacteria. In addition, bacterial lysis can augment the release of therapeutic macromolecules from the bacteria (Jain and Mekalanos, 2000), thus potentially increasing the efficacy of the bacterial vector.

The discovery of other remotely controllable promoters will eventually give rise to the construction of multifunctional tumor targeting bacteria which can be manipulated in several ways *in vivo*, thus allowing a safe, effective and individual tumor therapy.

Experimental procedures

Bacterial strains, plasmids and growth conditions

S. typhimurium strain SL7207 (*hisG*, Δ*aroA*) was kindly provided by Bruce Stocker (Hoiseth and Stocker, 1981). The coding sequence of the firefly luciferase (Fluc) gene from plasmid pGL3-basic (Promega) was placed under control of the P_{BAD} promoter regulator cassette, originally subcloned from Plasmid pBad18 (Guzman *et al.*, 1995), yielding plasmid pHL259 (pMB1 origin of replication). Similarly, gene *gfpmut2* encoding a bright variant green fluorescent protein (Cormack *et al.*, 1996) was placed under control of the P_{BAD} promoter, yielding either the high copy number plasmid pHL238 (pUC origin of replication) or the low copy number plasmid pHL239 (p15A origin of replication). The low copy plasmid pSL1 is a derivative of pSMART-LCKan (Lucigen) and harbours the *luxCDABE* operon originating from *Photorhabdus luminescens* (Winson *et al.*, 1998). *lux* was placed under control of the P_{BAD} promoter and integrated into the chromosome of strain SL7207, yielding strain SL7207::HL289. In brief, *lux* was linked to the P_{BAD} promoter and the cassette subcloned into a derivative of plasmid pUX-BF5 harbouring a Tn7 mini-transposon (Bao *et al.*, 1991), yielding plasmid pHL289. Transposon-mediated site specific integration into the chromosome of SL7207 was carried out according to the method of Bao et al.. Similarly, gene E of bacteriophage ΦX174 was placed under control of the P_{BAD} promoter and integrated into the chromosome of strain SL7207, yielding strain SL7207::HL260a. Bacteria were grown in Luria-Bertani medium (LB) supplemented with 100 µg ml-1 ampicillin, 30 µg ml-1 kanamycin, 30 µg ml-1 streptomycin, 15 g l-1 agar where appropriate. Bacteria were mostly grown at 37°C, in liquid medium with vigorous shaking.

Infection of J774A.1 mouse macrophages and addition of L-arabinose to cell culture

J774A.1 mouse macrophages (ATCC TIB-67) were grown in IMDM medium (Gibco) supplemented with 10 % (v/v) fetal calf serum, 2 mM l-glutamine and 10 mM HEPES. 2,5 x 10^5 cells were seeded in each well of a 6-well cell culture plate, incubated overnight and subsequently infected with *S. typhimurium* at a multiplicity of infection 50:1 (bacteria : cells). Plates were centrifuged for 1 min at 1000 g and incubated 30 min at 37°C. Then, cells were washed with medium and extracellular bacteria were killed by incubation with medium containing 50 µg ml-1 gentamycin (Biochrom). The medium was replaced after 1,5 hr with medium containing 10 µg ml-1 gentamycin. L-arabinose dissolved in medium was added at different concentrations into wells and incubation continued for another 4 hrs. Cells were washed twice with phosphate-buffered saline (PBS) and reporter gene expression was analyzed.

Flowcytometric analysis of bacterial GFP expression

Cultured bacteria or bacteria present within the lysate of infected J774A.1 mouse macrophages were analyzed by flow cytometry. Infected J774A.1 cells were first lysed in PBS containing 0,5 % (v/v) Triton X-100 and incubated at 37°C for 5 min. Then, an equal volume of PBS containing 2 % (v/v) fetal calf serum and 2 mM EDTA was added before samples were analyzed. Two color flow cytometry was applied in order to differentiate GFP expressing bacteria from autofluorescent cellular debris as described previously (Bumann, 2002). This method exploits the difference of orange/green emission ratios of GFP expressing *S. typhimurium* and cellular debris (Supplementary Fig. 1a,b). A threshold value was set to exclude bacteria expressing no GFP and background autofluorescence from measurements. An appropriate scatter gate was used to distinguish bacteria from large particles. Data were analyzed with WinMDI 2.8 software.

Scanning electron microscopy

Samples were fixed in growth medium by adding 5 % (v/v) formaldehyde and 2 % (v/v) glutardialdehyde for 1 hr at 4°C. After washing three times with cacodylate buffer (0,1 M cacodylate, 0,09 M sucrose, 0,01 M $MgCl_2$, 0,01 M $CaCl_2$, pH 6,9) bacteria were settled onto poly-L-lysine coated glass cover slips for scanning electron microscopy and fixed again with 2 % (v/v) glutardialdehyde in cacodylate buffer for 10 min at 25°C. After washing with TE buffer (10 mM TRIS, 1 mM EDTA) samples were dehydrated with a graded series of acetone (10, 30, 50, 70, 90, 100 %), each step for 15 min on ice. After critical-point drying with liquid CO_2 (Bal-Tec CPD 030) samples were sputter coated with a thin gold film (Balzers Union SCD 040). Samples were examined in a Zeiss field emission scanning electron microscope DSM 982 Gemini at an acceleration voltage of 5 kV applying the Everhart-Thornley SE-detector and the inlens detector at a 50:50 ratio. Images were stored on MO-disks and contrast and brightness was adjusted using Adobe Photoshop 6.0.

Determination of L-arabinose concentration in blood plasma

The concentration of L-arabinose in blood plasma of mice was enzymatically determined by the method of Melrose and Sturgeon (Melrose and Sturgeon, 1983). In brief, 200 µl blood were obtained by retro orbital bleeding and 25 units heparin sodium were added immediately. Samples were centrifuged at 10000 x g for 5 min and 50 µl of the supernatant were mixed with 830 µl of 0,1 mM Tris-HCl (pH 8,6) and 100 µl of 5 mM nicotinamide-adenine dinucleotide (ß-NAD, Sigma-Aldrich). Absorbance at 339 nm (A1) was measured using the Spectronic 401 photometer (Milton Roy) and subsequently 20 µl of 5 units ml-1 galactose dehydrogenase (GalDH, Roche) were added. After incubating the reaction mixture for 40 min at room

temperature, measurement (A2) was repeated. The concentration of L-arabinose was calculated from the change of absorbance (A2-A1) multiplied with factor 475,9.

Infection of tumor bearing mice and recovery of bacteria from tissues

BALB/c mice were inoculated subcutaneously at the abdomen with 10^6 cells of the colon adenocarcinoma cell line CT26 (ATCC CRL-2638). Mice bearing tumors of approxiately 4 - 6 mm diameter were intravenously injected with 5 x 10^6 cfu of bacteria suspended in phosphate-buffered saline (PBS). At day 3 post-infection (p.i.), 120 mg L-arabinose disolved in PBS were intraperitoneally administered to mice. At various time points after L-arabinose injection mice were sacrificed and tumors, spleens and livers were transferred into 3 ml of sterile ice-cold PBS containing 0,1 % (v/v) Triton X-100. Tissues were disrupted by using a Polytron PT3000 homogenizer (Kinematica). For determination of plasmid harboring bacteria, homogenates were serially diluted in PBS and plated with or without antibiotics.

Quantification of bacterial Fluc expression

To small samples of bacterial culture, cell lysates or tissues homogenates an equal volume of bacterial lysis buffer (50 mM Tris-HCl pH 8,3, 4 mM DTT, 20 % (v/v) glycerol, 2 % (v/v) Triton X-100, 2 mg ml-1 lysozyme) was added and the mixture incubated for 10 min at 25°C. Fluc activity of these samples was determined using the Luciferase Assay System (Promega) according to the manufacturer's instructions. In brief, 10 µl of sample was mixed with 100 µl of the Luciferase assay reagent (LAR) and subsequently the emission of light was measured during a 10 s time interval in a Lumat LB9507 luminometer (Berthold) as relative light units (RLU).

Non-invasive in vivo imaging of bioluminescence

Tumor bearing mice were intravenously injected with *S. typhimurium* harbouring either the *fluc* plasmid or the chromosomal *lux* cassette as described above. Luciferase expression was induced by intraperitoneal administration of 120 mg L-arabinose at day 3 p.i.. Mice were anaesthetized with isoflurane using the XGI-8 gas anesthesia system (Xenogen). Prior to image acquisition 3 mg luciferin (Synchem) dissolved in 100 µl PBS were injected intraperitoneally when appropriate. Images were obtained at consecutive time points thereafter using the IVIS-100 system (Xenogen) according to instructions of the manufacturer. The software Living image 2.5 (Xenogen) was used for image analysis and quantification of emission intensities.

Histology

Tumors were removed from sacrificed mice and snap frozen in Tissue-Tek OCT Compound (Sakura Finetek). Cryosections of 10 µm were cut with a with a microtome-cryostat (Cryo-Star HM560V, Microm) and placed onto slides. Slides were air dried at room temperature for 24 h and fixed in aceton at -20°C for 3 min. Slides were rehydrated in PBS, blocked with 50 µg ml-1 BSA and 1 µg ml-1 FcR blocker (rat-α-mouse CD16/CD32), and stained with the following reagents: polyclonal rabbit-α-*S.typhimurium* (Sifin), polyclonal goat-α-rabbit Fitc (Sigma), polyclonal goat-α-rabbit Cy3 (Jackson), Phalloidin Alexa-Fluor 594 (Molecular Probes) and DRAQ5 (Biostatus). After staining, the slides were washed and dried, mounted with mounting medium (Neomount, Merck) and analyzed using a laser scanning confocal microscope (LSM 510 META, Zeiss). Images were processed with LSM5 Image Browser (Zeiss) and Adobe Photoshop 7,0. For GFP-expressing bacteria, tumors were fixed overnight in 2,5 % (v/v) paraformaldehyde in PBS at 4°C. Afterwards the tumors were washed twice in 10% (v/v) sucrose in PBS at 4°C for 3 hrs. Snap freezing, cutting and staining was done as before, except fixation in acetone was

omitted. For paraffin sections, tumors were fixed in 10 % (v/v) paraformaldehyde and embedded in paraffin wax. 2 µm sections were mounted on glass slides and a Warthin-Starry silver staining was performed. Nuclei were counterstained with kernechtrot.

Figure legends

Fig. 1. Reporter gene expression of *S. typhimurium* after induction with L-arabinose in bacterial cultures or after phagocytosis by mouse macrophages.

A) *S. typhimurium* SL7207 harboring plasmids that encode GFP controlled by P_{BAD} were induced with 0.1 % (w/v) L-arabinose in bacterial cultures and analyzed 2 hrs after induction by flow cytometry. Open area shows uninduced bacteria, grey area indicates induced bacteria. B) bacteria harboring plasmids that encode Fluc controlled by P_{BAD} were induced as in a) and enzymatic activity of luciferase was determined from lysates 2 hrs after induction and calculated as relative light units (RLU) per colony forming unit (cfu). C,D) Both strains were used to infect J774A.1 macrophages. 2 hrs after infection, L-arabinose at indicated final concentrations was added. C) Bacterial GFP expression in cellular lysates was quantified by flow cytometry 4 hrs after induction. Values of the median GFP fluorescence intensity of bacteria were obtained from histograms shown in Supplementary Fig. 2B. D) Fluc expression by intracellular bacteria was quantified 4 hrs after induction as in B. Experiments were performed at least three times with similar results. b.t. - below detection.

Fig. 2. L-arabinose induced expression of Fluc by *S. typhimurium* in tumor bearing BALB/c mice.

A) Concentration of L-arabinose in blood plasma was measured at consecutive time points after intraperitoneal administration of 120 mg sugar. B) Colonization of host tissue by *S. typhimurium* bearing plasmids with inducible Fluc. Tissue homogenates were plated at consecutive time points during the course of induction. C, D) kinetics of L-arabinose induced Fluc expression by *S. typhimurium* in host tissues. C) Total Fluc activity from tissue homogenates was determined, and D) Fluc activity per

bacterial cell was calculated. For each time point three animals were used and experiments were performed three times with similar results.

Fig. 3. Non-invasive *in vivo* imaging of L-arabinose induced bacterial bioluminescence.

Tumor bearing mice were infected with either *S. typhimurium* harbouring the inducible *fluc* plasmid (A, B) or the chromosomal inducible *lux* cassette (C, D). 3 days p.i. mice were intraperitoneally injected with 120 mg L-arabinose. Images of anaesthetized mice were acquired either before (0 h) or after administration of L-arabinose with the IVIS-100 CCD camera system at indicated time points. In case of *fluc* bacteria, mice received luciferin substrate intraperitoneally at each time point immediately before imaging (A, B). Consecutive images of individual mice are presented (A, C). The intensity of bioluminescence emission was quantified for all mice of each group (B, D). Similar results were obtained in three independent experiments.

Fig. 4. Histology of tumors colonized by *S. typhimurium*.

A-F) Cryosections of CT26 tumors from infected mice were prepared 3 days p.i.. A) Low magnification overview of a *S. typhimurium* colonized tumor, bacteria are stained in green and cellular actin in red. B,C) Higher magnifications of the border region between viable cells and necrotic tissue. The white box in A) and B) indicates the area of enlargement in the subsequent picture. D-F). L-arabinose inducible GFP expression by tumor colonizing *S. typhimurium*. Bacteria harbor a plasmid encoding GFP under the control of P_{BAD}. Tumors were excised 6 hrs after administration of L-arabinose. GFP expressing bacteria are found in the border region between viable and necrotic tumor tissue (D) and inside the necrotic region (E), of which the

indicated segment is enlarged (F). Bacteria are stained in red, GFP-expressing bacteria are green and nuclei are stained in blue (D-F). G-I) Paraffin sections of a tumor colonized by *S. typhimurium.* Bacteria and cellular membranes are revealed by silver staining and nuclei are counterstained with Kernechtrot. In the necrotic region (G) and at the border between viable and necrotic tumor tissue (H) bacteria are mainly extracellular. In viable tissue of the tumor rim no bacteria were found (I). The insets show 2.5 fold magnifications of parts of each picture. White bars correspond to 100 µm in (A), 10 µm in (B-E) and black bars 20 µm in (G-I), respectively.

Fig. 5. L-arabinose inducible expression of the lysin gene E of bacteriophage ΦX174. The *S. typhimurium* strain harbors a chromosomal cassette encoding lysin gene E under control of P_{BAD}. A) Bacteria of this strain were grown in the absence or presence of 0.05 % (w/v) L-arabinose and the optical density (OD) of cultures was recorded. Dotted line indicates the time point of addition of the sugar. B) scanning electron micrographs were prepared of bacteria from an uninduced culture, or C) of bacteria 90 min after addition of L-arabinose. The white bars correspond to 1 µm. D) bacteria of this lytic strain were administered to tumor bearing mice and 2 days p.i. groups of mice received intraperitoneally either PBS or 120 mg L-arabinose, followed by a second dose after an 6 hr interval. After additional 6 hrs, tumors, spleens and livers were obtained and bacteria were estimated from homogenates by plating (D, each group n=7). Similar results were obtained in three independent experiments.

Acknowledgements

We would like to thank Regina Lesch and Susanne zur Lage for expert technical help, Dr. Reinhard von Wasielewski for help with the histology and Dr. Nelson Gekara for critical reading of the manuscript. This work was supported in part by the German Research Council (DFG), the Deutsche Krebshilfe and the Bundesministerium für Wirtschaft und Technologie (BMWi).

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