**Abstract**

A tumor promoting role of macrophages has been described for a transgenic murine breast cancer model. In this model tumor-associated macrophages (TAMs) represent a major component of the leukocytic infiltrate and are associated with tumor progression. *Shigella flexneri* is a bacterial pathogen known to specifically induce apoptosis in macrophages. To evaluate whether *Shigella*-induced removal of macrophages may be sufficient for achieving tumor regression we have developed an attenuated strain of *S. flexneri* (M90T*aroA*) and infected tumor bearing mice. Two mouse models were employed, xenotransplantation of a murine breast cancer cell line and spontaneous breast cancer development in MMTV-HER2 transgenic mice. Quantitative analysis of bacterial tumor targeting demonstrated that attenuated, invasive *Shigella flexneri* primarily infected TAMs after systemic administration. A single i.v. injection of invasive M90T*aroA* resulted in caspase-1 dependent apoptosis of TAMs followed by a 74% reduction in tumors of transgenic MMTV-HER2 mice 7 days post infection. TAM depletion was sustained and associated with complete tumor regression. These data support TAMs as useful targets for antitumor therapy and highlight attenuated bacterial pathogens as potential tools.

**Introduction**

Ever since the American surgeon Coley described streptococcal infection as a potential cure of cancer [1], other bacteria have been explored and were shown to infiltrate, replicate and accumulate in tumors [2,3]. For some extracellular bacteria, such as genetically modified obligate anaerobe *Clostridium novyi*, an anti-tumor effect was observed [4]. Other extracellular bacteria such as *Escherichia coli* accumulated in tumor tissue, induced some inflammatory responses, but failed to confer protection [5]. Facultative intracellular bacteria such as *Salmonella* have also been assessed for tumor therapy and their intratumoral accumulation was studied using different technologies, albeit beyond cellular resolution [6,7,8,9]. In most syngenic experimental models the therapeutic effect was moderate, whereas in xenograft models a more pronounced effect was described [7,9,10,11,12]. It was speculated that induction of an inflammatory response was mediating the anti-tumor effect.

In contrast to extracellular bacteria, intracellular bacteria can deliver DNA into eukaryotic cells [13]. Therefore, intracellular bacteria could be employed to deliver toxins or prodrug converting enzymes directly into tumor cells. Yet, no quantitative information on the distribution of intracellular bacteria in different stromal versus tumor cells is available even though such data are key to the design of effective therapeutic regimens [14].
Tumors consist of a complex mixture of transformed cells and stroma cells [15]. In many tumors, tumor-associated macrophages (TAMs) represent a major component of the leukocyte infiltrate [16]. High macrophage numbers have been reported in breast, ovarian, prostate, pancreatic and cervical cancers and are associated with poor prognosis [16,17]. Some authors have characterized TAMs as macrophages expressing protumoral functions, including promotion of tumor angiogenesis, metastasis, matrix remodelling and suppression of adaptive immunity [16,18,19,20]. Similar results have recently been obtained for tumor associated neutrophils [21].

Removal of macrophages or neutrophils reduced the rate of tumor progression in murine tumor models [21,22]. Evidence suggests that TAMs are tumor-educated macrophages that appear to have defective production of reactive oxygen and nitrogen intermediates and are impaired in phagocytic activity [16]. For normal macrophages it is known that they are a primary target of virulent Shigella flexneri [23]. Shigella flexneri infection triggers caspase-1 activation leading to apoptosis and processing of IL-1 and IL-18 [23].

To analyse the distribution of shigellae after i.v. application in tumor models with high numbers of macrophages (Supporting Information S1: Fig. S1), we quantified the numbers of bacteria in the extracellular space or within tumor cells, distinguishing between the macrophages and non-macrophages. Here we show that TAMs were the primary target of invasive shigellae in a 4T1 tumor xenograft - and a transgenic MMTV-HER2 - breast cancer model. Metabolically attenuated, invasive Shigella flexneri were almost exclusively found intracellularly, whereas a non-invasive S. flexneri mutant predominantly located extracellularly. Invasive shigellae, but not non-invasive shigellae, were able to activate caspase-1 and induce apoptosis in TAMs in both breast cancer models. Shigellae induced apoptosis caused a substantial depletion of TAMs, which was correlated with a pronounced and long-lasting therapeutic effect in the 4T1 breast cancer model. Finally, apoptosis induction by Shigella was confirmed ex vivo with freshly isolated human TAMs. Taken together the data suggests that invasive bacteria capable of inducing apoptosis in macrophages might represent a novel and promising therapeutic approach for a macrophage-targeted tumor therapy.

Materials and Methods

Ethics Statement

All animals experiments were carried out in accordance with protocols approved by the Regierung von Unterfranken, Germany.

Media, Chemicals and Other Reagents

Bacteria were grown on trypticase soy broth, trypticase soy agar (Becton Dickinson), LB broth, LB Agar (Sigma-Aldrich) or BHI (Becton Dickinson). Ampicillin-, chloramphenicol-, and kanamycin-resistant transformants were selected on agar containing the respective antibiotic at 100, 25, and 30 mg/ml. L-arabinose (Sigma-Aldrich) was used at 1 mM for gene expression induction. TSA containing 100 mg/l of Congo red dye was used to select Cr+ clones of S. flexneri [24]. Oligonucleotides (Eurofins MWG Operon), Enzymes (Fermentas) and Taq polymerase (Biotherm, Genecraft) were used according to manufacturers’ instructions. QiaGen products were used to isolate plasmid DNA, gel-purify fragments, or purify PCR products.

Bacterial Strains, Growth Conditions

The S. flexneri serotype 5a strains used in this study are the wt M90T (streptomycin (Sm) resistant) and its noninvasive variant BS176 [lacking the virulence plasmid pWR100] [25,26]. Strains containing pKD46 and pCP20 were incubated at 30°C unless otherwise noted.

Generation of Shigella aroA-Mutants

Linear DNA containing antibiotic resistance genes were prepared from the plasmids pKD3 according to the method described by Datsenko and Wanner [27]. Briefly, a PCR-product was generated by using primers AroA_up (GGGGTTTTTTA-TTTCGTGTAGAGATGTTGCTAGCTGGAACTGTT-AGGGCTGGAGCTGTTTC) and AroA_down (GGCCGGTACA-TTGGGATCAAGATCTGCTGGTATCTGACATA-GAATATCCCTCCCTTA) with 36 nucleotide extensions that are homologous to the AroA-gene. As a template, the pKD3 plasmid, which carries a chloramphenicol resistance gene flanked with FRT sites, was used. The PCR-product was transformed into electrocompetent Shigella flexneri BS176, carrying the recombine encoding plasmid pKD46. In positive clones, the resistance cassette was eliminated using the flipase encoding helper plasmid pCP20 and finally the temperature sensitive helper plasmid was eliminated.

The resulting strain Shigella flexneri BS176ΔaroA (termed BS176Aaro) was transformed with the virulence plasmid pWR100 isolated from S. flexneri M90T and pCP20 as helper plasmid. After selection and elimination of the helper plasmid the mutant S. flexneri BS176ΔaroA pWR100 was obtained. The mutant carries the main feature of the virulent strain M90T and therefore was termed M90TΔaroA (M90TA).

Determination of CFU and Number of Infected Cells

CFUs were determined by plating serial dilutions in PBS containing 0.1% Triton-X (Roth) and plating on LB agar plates for shigellae strains. Colonies were counted after incubation overnight. For determination of the number of infected cells, serial dilutions were made in PBS, mixed with 5ml of 50°C heated SeaPlaque Agarose (Biozynm Scientific) and dropped on LB agar plates. The number of bacterial colonies was counted after overnight incubation. Every colony marks an infected eukaryotic cell.

HeLa Cell Invasion Assays and Survival Assay

Invasion and survival in HeLa (American Type Culture Collection, ATCC; CCL-2) cells was assessed using a modified gentamicin protection assay as previously described [28]. Briefly, six-well plates were seeded with 2 ml of HeLa cell suspension adjusted to 2×10⁶ cells/ml and incubated overnight to reach 90% confluency. Log-phase (OD₆₀₀ 0.6–0.8) cultures of bacteria were added at an estimated MOI of 100 and plates were incubated at 37°C for 1 h. Subsequently plates were washed three times with PBS and then incubated with DMEM containing gentamicin (100 µg/ml) for 1 h at 37°C in 5% CO₂. HeLa cells were lysed in a 0.1% Triton X-100 solution for 10 min at different time points and CFU was determined.

Cell to Cell Spreading Assay

To determine cell to cell spread with a growth attenuated strain a new assay was employed. HeLa cells (7×10⁶) grown in 6-well tissue culture plates were infected at a MOI of 500 for 1 h and then washed twice with PBS. Infected cells were irradiated at 20 Gray. Subsequently, a monolayer of uninfected HeLa cells grown
in 6 well plates was incubated with the irradiated *Shigella*-infected HeLa cells in a ratio of 70:1 for 2 h, 0 h and 12 h. After 1 h incubation with 100 μg/ml gentamicin, the concentration of gentamicin was reduced to 10 μg/ml. The number of infected cells was determined as described above.

**Analysis of Caspase-1, Caspase-3, IL-1β, IL-18 and PARP Processing by Western Blot**

Up to 1×10^6 cells were washed twice with PBS and lysed in 120 μl of 2x Laemmlı buffer at 100°C. 10-30 μl of lysates were separated by SDS-PAGE and transferred onto nitrocellulose membranes (Schleicher & Schuell). After 1 h blocking in 5% skimmed milk (Applichem), the membranes were incubated with the primary antibodies (anti-caspase-1/ICE, Sigma); anti-cleaved caspase-3 antibody (NEB), anti-IL1α antibody (BioVision), anti-IL1β antibody (BioVision), anti-cleaved PARP antibody (BD Pharmingen), anti-GAPDH antibody (Chemicon international) or anti-β-actin antibody (Sigma-Aldrich) diluted in 5% skimmed milk overnight at 4°C. After washing, the blot was incubated with peroxidase-conjugated secondary antibody (GE Healthcare). Detection was performed using the ECL kit (Amersham Biosciences) and exposure on X-ray film (Kodak).

**Mice and Tumor Inoculation**

Six- to eight-week-old female mice were injected subcutaneously with either 1×10^4 4T1 cytokeratin positive murine epithelial mammary cancer cells (ATCC, CRL-2539), 1×10^6 B78-D14 [29] melanoma cells or 1×10^6 P815-PSA [30] mastocytoma cells each resuspended in 50 μl PBS. Injections were done on both sides of the shaved abdomen. Balb/c, C57/BL6, DBA-2 mice were obtained from Harlan Winkelmann GmbH, Germany. Transgenic MMTV-HER2/new FVB mice were obtained from Jackson Laboratory [31]. All animals were housed at the MSZ animal care facility and experiments were carried out in accordance with protocols approved by the Regierung von Unterfranken, Germany.

**Histological and Immunohistochemical Analysis of Tumors**

Tumors grown to approximately 1.5 cm in diameter were excised aseptically. The tumors were formalin-fixed, sectioned, and stained with hematoxylin and eosin (Sigma-Aldrich). To identify macrophages at the tumor site, tissues were fixed in 4% buffered paraformaldehyde for one day, paraffin-embedded, and processed for sectioning. Sections were immunostained using the pan-macrophage antibody anti-F4/80 rat monoclonal antibody (Acris Antibodies) and specific reactivity was detected using a peroxidase-conjugated secondary antibody (GE Healthcare). Detection limits for spleen cells (not shown) are usually 10 fold lower due to higher cell numbers.

**FACS Analysis**

The relative amount of macrophages in tumor tissues was determined by FACS. After blocking the Fcγ receptor (anti-CD16/32, BD Pharmingen), cells were stained with FITC-anti-mouse CD11b (Milenyi-Biotec; 10 minutes at 4°C), phycoerythrin (PE)-anti-mouse Gr-1 (Milenyi-Biotec; 10 minutes at 4°C) or PE-anti-mouse F4/80 (Acris Antibodies; 30 minutes at 4°C). FITC or PE coupled IgG2B antibody (RD Systems) was used as isotype control. Total cell numbers were assessed in a FSC/SSC live gate. Flow cytometric analysis was performed on a FACS Calibur (BD Immunocytometry Systems) according to the manufacturer’s instructions.

**Efficacy Studies**

Therapeutic efficacy of *Shigella* infection on tumor growth was explored in 28 six- to eight-week-old female Balb/c mice bearing 4T1 tumors. Tumor growth was determined every two days with a caliper. At day 14 post cell implantation, mean tumor volume had reached 170 mm³ and 24 mice were randomized into three groups. A total amount of 1×10^6 M90TaroA4, BS176aroA4 or 100 μl PBS were injected into the lateral tail vein of the tumor-bearing mice. Tumor size was monitored every second day. Tumor volume was calculated with the formula V = π/6*a*b² (a>b). 31 days after tumor cell implantation the nude and the BS76aroA4 group (tumor volume of approx. 4000 mm³) and two M90TaroA4 mice (macrescopic comparison of tumors, data not shown) were sacrificed. On day 62 post tumor cell implantation M90TaroA4-infected mice were sacrificed to determine CFU in tumor, liver, spleen and for FACS and histological analysis.

**Statistical Analysis**

Statistical analysis was performed using Graph Pad Prism 4 (GraphPad Software). Data were compared and analyzed for statistical significance by using a two-tailed Student’s t-test whereas p<0.05 was considered as significant.

**Results**

**Generation and In Vitro Characterization of Attenuated Shigella Strains**

For infection, *S. flexneri* serotype 5a strains M90T and BS176 were used. BS176 is a variant of M90T lacking the virulence
plasmid [32]. An auxotrophic and attenuated strain for animal studies was constructed, by chromosomal deletion of the ara4-gene locus. Isogenic virulent and avirulent strains were generated by attenuating Shigella flexneri strain BS176 to yield BS176△aroA followed by transfer of the virulence plasmid pWR100 [23]. The resulting mutant was called M90T△aroA.

The strains were characterized with respect to extracellular and intracellular growth, adhesion, invasion and cell-to-cell spread in vitro (Fig. 1). The attenuated mutants had a reduced maximal growth rate in LB medium compared to the parental strains with functional ara4-genes (Fig. 1A). With respect to association and invasion, S. flexneri M90T△aroA was indistinguishable from the M90T strain (Fig. 1B). Consistent with the behaviour of other ara4 deleted mutants the intracellular replication was reduced (Fig. 1C). To determine the cell-to-cell spread, a spreading assay was applied, which is independent of intracellular replication (see material and methods section). S. flexneri M90T△aroA was actively spreading as a 6- or 17-fold increase of infected cells was seen at 6 vs 12 hours post coincubation (Fig. 1D). In contrast the isogenic control BS176△aroA was only marginally active (Fig. 1D).

The capacity of S. flexneri M90T△aroA to induce both caspase-1 cleavage (Fig. 1E) and apoptosis (Fig. 1F) was confirmed in J774A.1 macrophages by western blot analysis (Supporting Information S1: Fig. S2). Apoptosis induction by S. flexneri M90T△aroA was caspase-1 dependent, as the caspase-1 specific inhibitor YVAD-CHO fully blocked caspase-1 and PARP processing (Fig. 1E).

Infection of TAMs by Shigella In Vivo

In the next set of experiments, 1×10⁶ invasive S. flexneri M90T△aroA or non-invasive BS176△aroA derivative were applied i.v. to 4T1 xenografted Balb/c or tumor-bearing transgenic MMTV-HER2 mice. After tumor removal, single cell suspensions were prepared and treated with or without gentamicin to determine the ratio of extra and intracellular bacteria. Parts of the tumor cell suspension were separated by MACS into a TAM fraction and TAM-depleted fraction using an F4/80 antibody (Supporting Information S1: Fig. S3,4,5). Supporting Information S1: Fig. S3,4 also depicts the phenotypic characterization of the TAMs. 83% of CD11b positive cells were F4/80 positive (Supporting Information S1: Fig. S3). The F4/80 positive population is DC-M negative, MHC class II low and Gr-1 negative (Supporting Information S1: Fig. S4). The number of infected cells was determined by plating in L-TOP agar.

Similar to observations with other bacterial strains, both invasive and non-invasive Shigella accumulated and persisted in the tumor after i.v. infection, whereas they were rapidly eliminated in the spleen (Fig. 2A, C). However, both strains showed marked differences with respect to their intra-tumoral distribution. In the 4T1 tumor model, gentamicin treatment revealed that 6 h after infection the invasive S. flexneri M90T△aroA almost exclusively located intracellularly, whereas only 2% of the non-invasive derivative were located intracellularly (Fig. 2A). Seven days after application invasive Shigella reached total CFU levels of up to 6×10⁶ per tumor. Unexpectedly, the invasive strain was predominantly located in TAMs (≥10 fold enrichment, Fig. 2C) even though M90T△aroA readily infected epithelial 4T1 cells in culture (Supporting Information S1: Fig. S1). In case of the non-invasive BS176△aroA, only a small amount of bacteria were found that were restricted to macrophages 6 h p.i. (Fig. 2D), in line with previous reports [5]. Seven days after infection, non-invasive shigellae remained restricted to TAMs and were at the limit of detection (Fig. 2A). In contrast the ratio of invasive bacteria in TAMs vs non-TAMs was decreased at seven days p.i. presumably due to cell to cell spreading as observed in cell culture (Fig. 1E,F).

The predominant localization in TAMs was not due to higher CFU counts per cell compared to non-macrophages, as the infectious center assay led to the same results (Fig. 2B, D). The selective targeting of invasive shigellae to TAMs was also observed in tumor-bearing MMTV-HER2 transgenic mice (Fig. 2E, F).

Taken together, non-invasive BS176△aroA shigellae were mainly found extracellularly despite the presence of large numbers of macrophages in two different breast cancer models, whereas invasive M90T△aroA shigellae almost exclusively targeted TAMs.

Apoptosis Induction in TAMs

To examine whether the apoptosis induction observed in the established macrophage cell line J774A.1 in culture also occurred in TAMs in vivo, tumor bearing mice were infected. After infection with invasive M90T△aroA shigellae, caspase-1 activation (Fig. 3,4) was detectable in total cells and macrophage fractions of tumors taken 6 hours after infection. At seven days p.i. caspase-1 activation was exclusively seen in the macrophage fraction. Caspase-1 activation at either timepoint was invariantly associated with IL-18 and IL-1β processing (Fig. 3B,C) in addition to processing of the effector caspase-3 and PARP cleavage (Fig. 3A,D), a hallmark of apoptosis. In contrast the non-invasive BS176△aroA did not induce apoptosis. Apoptosis induction was only observed in cellular fractions, where shigellae were located intracellularly and that contained TAMs. Similar data were obtained for transgenic MMTV-HER2 mice (Fig. 3F). In both mouse models TUNEL staining in tumor tissue confirmed caspase-3 processing and PARP cleavage data (Supporting Information S1: Fig. S8).

Next we investigated whether apoptosis induction in vivo was associated with depletion of macrophages. Figure 4 shows that M90T△aroA, but not the avirulent shigellae reduced the relative content of TAMs in tumors from xenografted Balb/c mice by at least 50% seven days p.i. (Fig. 4F). In transgenic mice a 74% reduction was achieved by M90T△aroA but not control shigellae infection at the seven day timepoint (Fig. 4B). This result cannot be explained by differences in tumor sizes between animals (Supporting Information S1: Fig. S9) and therefore strongly suggests that infection with invasive bacteria induced the depletion of TAMs. In addition, the reduction of macrophages is not observed in spleens of infected animals suggesting that the reduction is confined to tumor tissues (data not shown).

Histological examination of non-infected (Fig. 4C left panels), BS176△aroA (Fig. 4C, middle panels) and M90T△aroA (Fig. 4C right panels) infected mice by anti-F4/80 staining confirmed the substantial reduction of macrophages. Additionally the IHC experiments revealed massive infiltration of inflammatory cells (anti-CD163 staining) in 4T1-tumors upon infection with invasive but not non-invasive Shigella (Fig. 4C). Furthermore, cytokeratin staining of tumors derived from M90T△aroA-infected mice, which identifies pockets of 4T1 tumor cells showed reduced content of cytokeratin positive cells. This result indicates that the depletion of macrophages is associated with a reduction of tumor cells as previously observed by other approaches targeting macrophages [19,33,34]. In the non-infected and BS176△aroA-infected tissue normal tumor structure was observed.

Tumor Regression by Depletion of TAMs

To investigate whether this substantial reduction in macrophage numbers and marked inflammation induced by S. flexneri M90T△aroA is associated with a therapeutic effect, bacteria were applied to tumor-bearing Balb/c mice and tumor growth was
Figure 1. *In vitro* characterization of *aroA* mutants. (A) Determination of extracellular growth rate in LB medium. (B) Invasion assay (gentamicin protection assay). CFUs are shown relative to the wt M90T. (C) Intracellular replication rate in HeLa cells. (D) Cell to cell spread in HeLa cells assessed by L-Top agar assay. Western blot analysis for caspase-1 activation (caspase-1*) (E) and apoptosis induction was performed in J774A.1 Macrophages (PARP cleavage - cPARP) (F). Bars represent means +/- SD of three different experiments. **P < 0.001.** BS176: *Shigella flexneri* BS176; BS176Δ: *Shigella flexneri BS176::aroA*; M90T: *Shigella flexneri* M90T; M90TΔ: *Shigella flexneri M90T::aroA*.

doi:10.1371/journal.pone.0009572.g001
Figure 2. *Shigella flexneri* M90T ΔaroA predominantly targeted TAMs *in vivo*. Determination of CFU/cell number (A, C, E) and infected cells/cell number (B, D, F) of separated tumor cells and spleen cells 6 h and 7d after i.v. infection with $1 \times 10^6$ bacteria of tumor-bearing 4T1 (A–D) and transgenic MMTV-HER2/neu mice (E, F). Corresponding raw data of CFU and infected cell counts are shown in Supporting Information S1: Fig. S6 and Supporting Information S1: Fig. S7. + indicates gentamicin treatment. Dashed lines indicate detection limits. All results shown are mean ± SD of values from three mice per experiment; ** P < 0.01, *** P < 0.001. BS176Δ: *Shigella flexneri* BS176 ΔaroA; M90TΔ: *Shigella flexneri* M90TΔ arA

doi:10.1371/journal.pone.0009572.g002
assessed (Fig. 5). Infection with *S. flexneri* BS176 ΔaroA resulted in a small, albeit significant reduction of tumor growth in comparison to the control animals. In contrast, a single i.v. infection with *S. flexneri* M90T ΔaroA resulted in complete removal of tumor tissue (Fig. 5). 48 days p.i. the tumor showed very low macrophage (<4%) numbers and bacteria were not detectable (Fig. 5). Histological examinations of the tumor tissue 68 days after xenografting showed persistent reduction of macrophage numbers...
Discussion

In this work we have demonstrated that invasive \textit{S. flexneri} preferentially target TAMs after i.v. infection in two different breast cancer models. The growth-attenuated virulent mutant M90T\textit{aroA}, but not the non-invasive mutant BS176\textit{aroA}, was exclusively located intracellularly at all time points examined. \textit{Mutant M90T\textit{aroA}}, but not BS176\textit{aroA}, induced caspase-1 processing and apoptosis in TAMs leading to a long-lasting reduction of TAMs. The reduction of TAMs was accompanied by a complete tumor regression in a 4T1 breast cancer model and removal of transformed cells in the residual stroma. In contrast to bacterial tumor therapies that use extracellular bacteria, targeting TAMs with invasive shigellae was effective despite relatively low numbers of bacteria within the tumor. Finally, for a different gynaecological cancer type of human origin, human ovarian carcinoma, we could show \textit{ex vivo} that infection of TAMs from ascites with \textit{S. flexneri} M90T\textit{aroA} induced caspase-1 activation and PARP cleavage (Supporting Information S1: Fig. S10).

Experimental tumor therapy by infection with extracellular bacteria has a long history \cite{3}. Recent observations with \textit{E.coli} strains suggested that these bacteria were able to induce tumor-targeted inflammatory responses but were ineffective in killing tumor cells \cite{5}. The non-invasive strain BS176\textit{aroA} showed a similar behavior, including a marginal, albeit significant, therapeutic efficacy. Bacterial tumor therapy is commonly thought to be based on the induction of an inflammatory response at the site of the tumor as a result of the accumulation of extracellular bacteria at this immunologically privileged site \cite{4,11,35}. Whereas these bacteria remain extracellular at all times \textit{S. flexneri} M90T\textit{aroA} actively invades cells. This data confirm previous reports which have described phagocytosis defects in TAMs \cite{16,19}.

\(\text{Figure 4. Infection with Shigella flexneri M90T.\textit{aroA} results in a decrease in TAMs number.}\) 7d after iv infection of tumor bearing Balb/c (A) and MMTV-HER2 FVB mice (B) tumors were analysed for F4/80 positive cells by FACS to determine the relative amount of TAMs. Bars represent means +/- 5D of four tumors analyzed per group, ** P<0.01, *** P<0.001. (C) Histology of tumor tissue from non-infected, BS176\textit{Δ} or M90T\textit{Δ} infected mice. In each case left panels represent antibody controls. Scale bars represent 50 μm. BS176\textit{Δ}: \textit{Shigella flexneri} BS176.\textit{aroA}; M90T\textit{Δ}: \textit{Shigella flexneri} M90T.\textit{aroA}; CK: Cytokeratin.

doi:10.1371/journal.pone.0009572.g004

(anti-F4/80 staining) and complete depletion of 4T1 tumor cells (anti-cytokeratin staining) (Fig. 5B).
Figure 5. M90TΔ, but not BS176Δ, completely blocked tumor growth after i.v. infection of 4T1 tumor-bearing mice. (A) 14 days after tumor cell inoculation $1 \times 10^6$ bacteria were applied i.v. to 8 mice per group. Non-infected and BS176Δ infected mice were sacrificed 31 days after tumor cell inoculation. At day 62, M90TΔ mice were killed and CFU and macrophage numbers were determined. ** $P<0.01$, *** $P<0.001$. (B) Histology of tumor tissue from M90TΔ-infected mice. Left panels represent antibody controls. Scale bars represent 50 μm. BS176Δ; Shigella flexneri BS176ΔaroA; M90TΔ; Shigella flexneri M90TΔaroACK; Cytokeratin. doi:10.1371/journal.pone.0009572.g005
In our experiments, the profound reduction of TAMs was associated with complete tumor regression [36]. The simplest interpretation of these data would be that TAMs are required to support tumor growth. However as we observed a massive infiltration of inflammatory cells simultaneous with bacterial infection and TAM depletion inflammation might also contribute to tumor regression. The extracellular BS176ΔaroA mutant did not induce caspase-1 processing and thus did neither induce IL-1β nor IL-18 activation nor deplete TAMs. Therefore it is likely that for the induction of the inflammatory response by M90TΔaroA targeting of macrophages is also a necessary factor. However the low dose bacteria found under these conditions makes it difficult to come up with a firm conclusion in this respect. As neutrophils have been described to be potential mediators of inflammation induced tumor regression [21] it may be interesting in the future to determine whether M90TΔaroA can induce neutrophil infiltration in the presence or absence of TAMs.

Targeting TAMs as a strategy for bacterial tumor therapy may have the advantage that a stable cell population is attacked as opposed to the phenotypically unstable tumor cell population, that may quickly give rise to resistant cells. On the other hand macrophages found in tumors may potentially stimulate or inhibit tumor growth. It will therefore be important in the future to develop markers that differentiate between TAM populations and allow identification of tumors where TAM removal may be beneficial [37,38]. Although at first sight *Shigella* would seem to be an unlikely candidate as a therapeutic agent based on its pathogenicity, the fact that attenuation was readily achieved and that a small number of bacteria at the tumor site was sufficient to induce a dramatic anti-tumor effect suggests that further investigation is warranted. At least in our experiments we did not observe signs of overt toxicity, albeit mice cannot be considered as a suitable model to assess toxicity of *shigellae*.

In summary, we suggest that targeting TAMs using attenuated *S. flexneri* is a promising option for future tumor therapy. Further studies are required with respect to the safety of the *S. flexneri* mutant and the efficacy of tumor targeting in humans. Furthermore, other intracellular bacteria like *Salmonella enterica* or *Listeria monocytogenes* might be suitable for a macrophage targeted bacterial tumor therapy.

**Supporting Information**

**Supporting Information S1**

Found at: doi:10.1371/journal.pone.0009572.s001 (15.15 MB DOC)

**Acknowledgments**

Plasmids were kindly provided by Christian Andersen, Würzburg. The authors wish to thank S. Roos and B. Oberwallner for technical assistance, M. Becker, R. Reuten, E. Zanucco and C. Korn, T. Dogan, S. Tripschuch and H. Drexler for their support and helpful discussion.

**Author Contributions**

Conceived and designed the experiments: MH CH BB IG WG URR JF. Performed the experiments: KG MH. Analyzed the data: KG MH AG BB IG WG URR JF. Contributed reagents/materials/analysis tools: JW. Wrote the paper: KG MH CH JF.

**References**

1. Coley WB (1991) The treatment of malignant tumors by repeated inoculations of crysopelas. With a report of ten original cases. 1893. Clin Orthop Relat Res. pp 3–11.

2. Yu YA, Shahabang S, Timiajaya TM, Zhang Q, Reltz BM, et al. (2004) Visualization of tumors and metastases in live animals with bacteria and vaccinia virus encoding light-emitting proteins. Nat Biotechnol 22: 313–320.

3. Van Mellaert L, Barbé S, Anne J (2006) Clostridium spores as anti-tumour agents. Trends in Microbiology 14: 190–196.

4. Agravat NV, Bettegowda C, Cheong I, Geschwind JF, Drake CG, et al. (2004) Bacterial therapy can generate a potent immune response against experimental tumors. Proc Natl Acad Sci U S A 101: 13172–13177.

5. Weibel S, Stritzker J, Eck M, Goebel W, Scalu AA (2008) Colonization of experimental murine breast tumours by *Echerichia coli* K-12 significantly alters the tumour microenvironment. Cell Microbiol.

6. Forbes NS, Munn LL, Fukumura D, Jain RK (2003) Sparse initial entrapment of tumor-associated macrophages in tumor progression and invasion. Cancer and Metastasis Reviews 25: 315–322.

7. Kasinskas RW, Forbes NS (2007) *Salmonella* typhimurium Lacking Ribose Chemoreceptors Localize in Tumor Quiescence and Induce Apoptosis. Cancer Res 67: 5188–5195.

8. Zhao M, Yang M, Ma H, Li X, Tan X, et al. (2006) Targeted Therapy with a *Salmonella* Typhimurium Leucine-Aminopeptidase Auxotroph Cures Orthotopic Human Breast Tumors in Nude Mice. Cancer Res 66: 7647–7652.

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

10. Carmeliet P, Jain RK (2000) Angiogenesis in cancer and other diseases. Nature 407: 249–257.

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

12. Vassaux G, Nitcheu J, Jezzard S, Lemoine NR (2006) Bacterial gene therapy strategies. The Journal of Pathology 208: 290–298.

13. Pilgrin S, Stritzker J, Schlen C, Kolb-Maurer A, Geginat G, et al. (2003) Bacteriofication of mammalian cells by *Listeria monocytogenes* improvement in and mechanism of DNA delivery. Gene Ther 10: 2036–2045.

14. Pawelek JM, Low KB, Bermudes D (1997) Tumor-targeting *Salmonella* as a Novel Anticancer Vector. Cancer Res 57: 4537–4544.

15. Mueller MM, Fuesing NE (2004) Friends or foes - bipedal effects of the tumour stroma in cancer. Nat Rev Cancer 4: 439–449.

16. Lewis CE, Pollard JW (2006) Distinct Role of Macrophages in Different Tumor Microenvironments. Cancer Res 66: 605–612.

17. Kurahara H, Shinchi H, Mataki Y, Maemura K, Noma H, et al. (2009) Significance of M2-Polarized Tumor-Associated Macrophage in Pancreatic Cancer. J Surg Res.

18. Low KB, Intensohn M, Le T, Platt J, Sodi S, et al. (1999) *Lipid A* mutant *Salmonella* with suppressed virulence and TNFalpha induction retain tumor-targeting in vivo. Nat Biotechnol 17: 37–41.

19. Mantovani A, Schioppa T, Porta C, Alleva P, Sica A (2006) Role of tumor-associated macrophages in tumor progression and invasion. Cancer and Metastasis Reviews 25: 315–322.

20. Qian B, Deng Y, Im JH, Muschel RJ, Zou Y, et al. (2009) A Distinct Macrophage Population Mediates Metastatic Breast Cancer Cell Extravasation, Establishment and Growth. PLoS ONE 4: e6562.

21. Friedlander ZG, Sun J, Kim S, Kapoor V, Cheng G, et al. (2009) Polarization of tumor-associated neutrophil phenotype by TGF-beta: "N1" versus "N2" TAN. Cancer Cell 16: 183–194.

22. Cheng H-L, Traik B, Tsai T-S, Liu H-S, Chan S-H, et al. (2002) Overexpression of c-met as a Prognostic Indicator for Transitional Cell Carcinoma of the Urinary Bladder: A Comparison With *p53* Negative Accumulation. J Clin Oncol 20: 1544–1550.

23. Zychlinsky A, Kenny B, Menard R, Prescot MV, Holland IB, et al. (1994) *IpaB* mediates macrophage apoptosis induced by *Shigella flexneri*. Mol Microbiol 11: 619–627.

24. Maurelli AT, Blackmon B, Curtis R, 3rd (1984) Temperature-dependent invasive ability of *Shigella flexneri*. Infect Immun 35: 852–860.

25. Sansonetti PJ, Kopecko DJ, Formal SB (1982) Involvement of a plasmid in the III secretion apparatus of *Shigella flexneri*. Mol Microbiol 38: 760–771.

26. Buchrieser C, Glaser P, Rusniok C, Nedjari H, D'Hauteville H, et al. (2000) *Listeria monocytogenes* and H. Drexler for their support and helpful discussion.

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

28. Densink KA, Wanner BL (2000) One-step inactivation of chromosomal genes in *Escherichia coli K-12* using PCR products. Proc Natl Acad Sci U S A 97: 6640–6645.

29. Elsinghorst EA (1994) Measurement of invasion by gentamicin resistance. *Listeria monocytogenes* invasion within tumors. Cancer Res 63: 5188–5195.

30. Fensterle J, Bergmann B, Yone, CLRP, Hotz C, Meyer SR, et al. (2008) Cancer Therapy by *Shigella flexneri* mutant and the efficacy of tumor targeting in humans. Further studies are required with respect to the safety of the *S. flexneri* mutant and the efficacy of tumor targeting in humans. Furthermore, other intracellular bacteria like *Salmonella enterica* or *Listeria monocytogenes* might be suitable for a macrophage targeted bacterial tumor therapy.

**Supporting Information**

**Supporting Information S1**

Found at: doi:10.1371/journal.pone.0009572.s001 (15.15 MB DOC)

**Acknowledgments**

Plasmids were kindly provided by Christian Andersen, Würzburg. The authors wish to thank S. Roos and B. Oberwallner for technical assistance, M. Becker, R. Reuten, E. Zanucco and C. Korn, T. Dogan, S. Tripschuch and H. Drexler for their support and helpful discussion.

**Author Contributions**

Conceived and designed the experiments: MH CH BB IG WG URR JF. Performed the experiments: KG MH. Analyzed the data: KG MH AG BB IG WG URR JF. Contributed reagents/materials/analysis tools: JW. Wrote the paper: KG MH CH JF.
31. Volpers C, Thirion C, Biermann V, Haasmann S, Kesess H, et al. (2003) Antibody-mediated targeting of an adenovirus vector modified to contain a synthetic immunoglobulin g-binding domain in the capsid. J Virol 77: 2093–2104.

32. Sansonetti PJ, Ryter A, Clerc P, Maurelli AT, Mounier J (1986) Multiplication of Shigella flexneri within HeLa cells: lysis of the phagocytic vacuole and plasmid-mediated contact hemolysis. Infect Immun 51: 461–469.

33. Lin EY, Gouon-Evans V, Nguyen AV, Pollard JW (2002) The Macrophage Growth Factor CSF-1 in Mammary Gland Development and Tumor Progression. Journal of Mammary Gland Biology and Neoplasia 7: 147–162.

34. Lin EY, Nguyen AV, Russell RG, Pollard JW (2001) Colony-Stimulating Factor 1 Promotes Progression of Mammary Tumors to Malignancy. J Exp Med 193: 737–740.

35. Hoiseth SK, Stocker BA (1981) Aromatic-dependent Salmonella typhimurium are non-virulent and effective as live vaccines. Nature 291: 238–239.

36. Zeisberger SM, Odermatt B, Marty C, Zehnder-Fjallman AH, Ballmer-Hofer K, et al. (2006) Clodronate-liposome-mediated depletion of tumour-associated macrophages: a new and highly effective antiangiogenic therapy approach. Br J Cancer 95: 272–281.

37. Lewis C, Murdoch C (2005) Macrophage responses to hypoxia: implications for tumor progression and anti-cancer therapies. Am J Pathol 167: 627–635.

38. Loesner H, Endmann A, Leschner S, Bauer H, Zelmer A, et al. (2008) Improving live attenuated bacterial carriers for vaccination and therapy. Int J Med Microbiol 298: 21–26.