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ORIGINAL ARTICLE

Invasion and destruction of a murine fibrosarcoma by *Salmonella*-induced effector CD8 T cells as a therapeutic intervention against cancer

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Abstract We have developed a new vaccination strategy by using the *Salmonella* type III secretion system (T3SS) to translocate heterologous antigens into the cytosol of host cells. This leads to an efficient antigen-specific CD8 T cell induction. Recently, we have demonstrated the use of *Salmonella*'s T3SS for the immunoprophylaxis of a solid tumor. The murine fibrosarcoma WEHI 164 was transfected with the DNA sequence encoding the MHC class I-peptide $p60_{217–225}$ from *Listeria monocytogenes*. In the present study, we used this tumor model to investigate the potential of vaccination with recombinant *Salmonella* in a therapeutic setting. BALB/c mice were subcutaneously challenged with WEHI-p60 cells. Simultaneously or 4 days later, these mice received either an orogastric or intravenous immunization with *Salmonella* translocating p60. Interestingly, 71–80% of the intravenously and 50–52% of the orogastrically immunized mice showed a complete tumor regression after 14 days. In addition, the distribution of tetramer-positive $p60_{217–225}$-specific CD8 T cell subpopulations in blood and tumor tissue was analyzed. Co-staining with CD62L and CD127 revealed that the frequencies of $p60_{217–225}$-specific effector and effector memory CD8 T cells in blood and in fibrosarcoma tissue were related to the kinetics of tumor regression. In summary, our study demonstrates that therapeutic vaccination with *Salmonella* leads to efficient induction of tumor-invading effector CD8 T cells that may result in significant tumor regression.

Keywords *Salmonella* vaccine · Murine fibrosarcoma model · Therapeutic anti-tumor immunity · Tumor-invading effector CD8 T cells

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Introduction

Clinical cancer immunotherapy has achieved encouraging, yet limited success. Tumors have evolved multiple mechanisms to evade the immune response, including antigen loss, downregulation of the major histocompatibility complex (MHC) and the production of immunosuppressive factors [10, 37, 39]. In addition, many tumors lack expression of co-stimulatory molecules critical for the activation of naïve T cells. Finally, tolerance mechanisms are operative in vivo to prevent T cell activation in response to tumor antigens that are expressed in many cases also in normal tissue. A strategy to circumvent part of these problems and to study particular aspects of cancer immunobiology includes the development of experimental tumor systems using tumors transfected with immunogenic model antigens of bacterial or viral origin [20, 22, 37]. Data compiled from both in vitro systems and animal models using either naturally occurring tumors or

transfected cancer cells clearly show that CD8 and CD4 T cells play a pivotal role in the effective eradication of tumors [11, 14, 19]. Thus, the majority of cancer immunotherapy efforts are devoted to the stimulation of T cellular immune responses.

Attenuated recombinant *Salmonella enterica* serovar Typhimurium has emerged as a promising delivery system for foreign vaccine antigens [21]. Upon close contact with the eukaryotic cell, a type III secretion system (T3SS) encoded by the "*Salmonella* pathogenicity island 1" mediates *Salmonella* invasion of the host cell, where the bacterium resides within *Salmonella*-containing vacuoles. The T3SS is designed to translocate *Salmonella* type III effector proteins directly into the cytosol of target cells [8]. Our laboratory has focused its research on the genetic manipulation of attenuated *Salmonella* strains to endow them with the ability for efficient induction of MHC class I-restricted immune responses [28, 32]. We have developed a new vaccination strategy by using the *Salmonella*-T3SS to translocate microbial antigens directly into the cytosol of antigen-presenting cells. The immunodominant p60 antigen from *Listeria monocytogenes* was fused to the defined N-terminal translocation domain of the type III effector molecule *Yersinia* outer protein E (YopE) [33]. Translocation and cytosolic delivery of the chimeric YopE/p60 protein into macrophages led to efficient MHC class I-restricted antigen presentation of the p60 nonamer peptide $p60_{217–225}$. As determined by enzyme-linked immunospot assay, mice orally vaccinated with a single dose of attenuated *Salmonella* expressing translocated YopE/p60 protein revealed high numbers of interferon-gamma (IFN-γ)-producing CD8 T cells reactive with $p60_{217–225}$. In a more recent study, we demonstrated that the use of *Salmonella*'s T3SS to induce antigen-specific cytotoxic T cells is also an attractive strategy to develop vaccines for the immunoprophylaxis of tumors [27].For these experiments, we established an experimental tumor model in BALB/c mice [27]. The murine fibrosarcoma cell line WEHI 164 [13] was stably transfected with DNA encoding the immunodominant listerial MHC class I-restricted nonamer epitope $p60_{217–225}$ [27]. In naïve mice, subcutaneous inoculation of these WEHI-p60 tumor cells resulted in progressive growth of a solid fibrosarcoma for approximately 14 days without inducing a measurable frequency of $p60_{217–225}$-tetramer-positive CD8 T cells. However, in vitro antigen presentation assays revealed that WEHI-p60 cells are able to present $p60_{217–225}$ via MHC class I molecules, although with a relatively weak efficiency. Thus, despite being transfected with an immunodominant bacterial antigen, this fibrosarcoma model resembles naturally occurring tumors that are often characterized by their weak immunogenicity. In further experiments, BALB/c mice received a single orogastric immunization with *Salmonella* that translocates YopE/p60 via its T3SS. Four weeks later, mice were challenged subcutaneously with WEHI-p60 tumor cells. In vivo protection studies revealed that 80% of these mice remained tumor free, whereas all animals of the non-vaccinated control group developed tumor growth. Taken together, our approach clearly demonstrated protection against a tumor in a prophylactic setting [27].

In this study, the potential of our vaccination strategy was evaluated with regard to a therapeutic intervention against cancer. Therefore, we used the above-described model and applied the YopE/p60 expressing *Salmonella* vaccine strain either simultaneously or 4 days after subcutaneous tumor injection.

Materials and methods

Plasmids, bacterial strains and growth conditions

The construction of plasmid pHR241 has been outlined in detail [33]. This low-copy number expression vector bears the genetic information for the translocated chimeric $YopE_{1–138}/p60_{130–477}$/M45 fusion protein under expression control of the *lac* promoter. M45 is derived from the E4–6/7 protein of adenovirus and its use for chimeric protein tagging has been described [33]. The above described plasmid was transformed into *Salmonella enterica* serovar Typhimurium strain SB824 by electroporation. Strain SB824 was engineered by introducing the *sptP::kan* mutant allele from strain SB237 into the Δ*aroA* strain SL3261 by P22HT*int* transduction [1, 32, 33]. Serovar Typhimurium was grown in Luria-Bertani medium supplemented with 0.3 M NaCl, pH 7.4, to allow expression of components and targets of the T3SS encoded by the *Salmonella* pathogenicity island 1, which mediates *Salmonella* invasion of host cells [33].

Stable transfection of fibrosarcoma cell line

Generation of WEHI-p60 cells has been outlined in detail [27]. To obtain WEHI-p60 cells, the $H\text{-}2^d$ fibrosarcoma WEHI 164 (ATCC # CRL-1751), a methylcolanthrene-induced tumor of BALB/c origin, was transfected with a double-stranded synthetic oligonucleotide (oligonucleotide A, 5′-CCGGTGCCACCATGAAATACGG TGTTTCTGT TCAAGACATTTGAG-3′; oligonucleotide B, 5′-GAT CCTCAAATGTCTT GAACAGAAACACCGTATTTCA TGGTGGCA-3′) encoding the $p60_{217–225}$-epitope, which was inserted into the mammalian expression vector pIRESneo3 (BD Biosciences, Heidelberg, Germany). Cells were maintained in RPMI 1640 supplemented with 10% fetal calf serum, L-glutamine, 2-mercaptoethanol, penicillin (100 U/ml), streptomycin (100 μg/ml) and G418 (400 μg/ml)

(Sigma, Deisenhofen, Germany). Slowly progressive in vivo growth was obtained by careful in vitro cell incubation to a density of maximum 70% and strict avoidance of medium acidification by continuous pH indicator controls [12]. No differences between WEHI 164 and WEHI 164-p60 growth could be observed in vivo or in vitro.

Generation and purification of H2-K^d tetramers

Tetrameric H2-K^d/$p60_{217–225}$ complexes were generated as previously described [2]. Briefly, recombinant H2-K^d heavy chain and β_2-microglobulin were expressed as insoluble inclusion bodies in *Escherichia coli* and were further purified. The H2-K^d heavy chain molecule was mutated to remove the transmembrane and cytosolic domain and to add a specific biotinylation site at the C-terminus. Purified proteins were refolded in vitro in the presence of high concentrations of synthetic peptides (Biosythan, Berlin, Germany) to form stable and soluble MHC/peptide complexes. Complexes were specifically biotinylated in vitro by adding the enzyme BirA, d-biotin and ATP. After further purification, biotinylated MHC/peptide complexes were multimerized with streptavidin-PE (SA-PE; Molecular Probes, Eugene, USA). Tetrameric complexes were purified by gel filtration and stored at 2–5 mg/ml at 4°C in phosphate-buffered saline (pH 8.0) containing 0.02% sodium azide, 1 μg/ml pepstatin, 1 μg/ml leupeptin and 0.5 ml EDTA.

Mice, in vivo tumor studies and immunization with recombinant *Salmonella*

Specific-pathogen-free female BALB/c mice, 6–8 weeks old, were purchased from Harlan–Winkelmann (Horst, Netherlands). For the experiments, animals were housed in groups of five mice under standard barrier conditions in individually ventilated cages (Tecniplast, Buguggiate, Italy). BALB/c mice received a subcutaneous injection of 5×10^6 WEHI-p60 cells in the flank on day 0. Mice given the fibrosarcoma cells were inspected on days 4, 5, 7, 10 and 14 for tumor growth and size. Tumor growth was measured by using a digital calliper to record maximum length and width. Values shown for tumor size (mm^2) are the product of these two parameters per animal averaged over the total number of mice in the respective group. Animal experiments were approved by the German authorities and performed according to the legal requirements. No apparent suffering of mice occurred after tumor inoculation and all animals behaved normally without any signs of pain or other disorders.

To evaluate the therapeutic potential of our *Salmonella*-based vaccination, four different immunization strategies were performed. The recombinant *Salmonella* vaccine strain SB824 (pHR241) expressing translocated YopE/p60 was applied intravenously (1×10^6 colony-forming units, CFU) on day 0 (immunization group A, simultaneous application with WEHI-p60 cells) or on day 4 after tumor injection (immunization group B). Mice of immunization group C received SB824 (pHR241) orogastrically (5×10^8 CFU) by using round-bottom gavage needles on day 0, whereas animals of immunization group D were vaccinated orogastrically on day 4 after subcutaneous tumor application. Control mice were immunized with plasmidless SB824 (immunization group S) or received phosphate-buffered saline (non-immunized, NI), respectively. Each experiment was performed at least twice with similar results. Complete tumor regression was defined by the calliper detection threshold of 3 mm × 3 mm. Observation of these mice for a period of three more months did not reveal any signs of local or systemic tumor relapse. As previously published [27], this immunization approach leads to the generation of sufficient amounts of p60-specific memory T cells, providing successful protection against tumor rechallenge 4 weeks post-vaccination.

Preparation of cells from blood and tumor tissue

Blood samples were collected on day 14 after tumor injection. Cells from blood were harvested by dissociation through a wire mesh and lysis of erythrocytes with ammonium chloride and were subsequently resuspended in RP10+, which consists of RPMI 1640 supplemented with 10% fetal calf serum, L-glutamine, HEPES (pH 7.5), 2-mercaptoethanol, penicillin (100 U/ml), streptomycin (100 μg/ml) and gentamicin (50 μg/ml).

On days 4, 5, 7, 10 and 14, fibrosarcoma tissue was removed from mice and single-cell suspensions were prepared by enzymatic digestion. Resected tumors were weighed, minced into small (1–2 mm^3) pieces with a scalpel and immersed in 10 ml of digestion mixture (5% FBS in RPMI 1640, 0.5 mg/ml collagenase A, 0.2 mg/ml hyaluronidase, type V and 0.02 mg/ml DNase I) per 0.25 g of tumor tissue. This mixture was incubated at 37°C for 45 min on a rotating platform. The resulting cell suspension was filtered sequentially through 70- and 40-μm cell strainers and washed with 5% FBS in RPMI 1640. Red blood cells were lysed by brief incubation in 0.15 M ammonium chloride solution.

MHC tetramer staining and FACS analysis

The $p60_{217–225}$-specific T cell populations from blood and tumor tissue were detected with phycoerythrin (PE)-conjugated, tetrameric MHC I/peptide complexes and concurrently stained for other surface molecules using directly conjugated monoclonal antibodies as described previously

[2, 3]. Briefly, cells were incubated with ethidium bromide monoazide for live/dead discrimination in FACS-staining buffer (phosphate-buffered saline, pH 7.45, 0.5% bovine serum albumin and 0.02% sodium azide). Subsequently, cells were stained with the above-mentioned MHC class I tetramer and surface markers for 1 h. The following monoclonal antibodies were used: anti-CD8α (clone 53-5.8, PharMingen, Heidelberg, Germany), anti-CD62L (clone Mel-14, PharMingen) and anti-CD127 (clone A7R34, eBioscience, San Diego, CA, USA). Cells were resuspended in staining buffer and fixed in 1% paraformaldehyde/phosphate-buffered saline (pH 7.45). Data were acquired on a FACSCanto (BD Biosciences, San Jose, CA, USA) and further analyzed with FlowJo software (TreeStar, Ashland, OR, USA).

Statistical analysis

The results are depicted as mean ± SD of triplicate determinations. Statistical significance of the results was checked with the non-parametric Mann–Whitney *U* test at the $*p < 0.01$, $**p < 0.005$ and $***p < 0.001$ significance level. Correlation analysis was performed with the non-parametric Kendall tau rank correlation test. All tests were performed using the SPSS (SPSS, Chicago, IL, USA) and Microsoft Office Excel 2007 software.

Results

Efficient reduction of fibrosarcoma growth after intravenous or orogastric vaccination with recombinant *Salmonella*

In this study, we wanted to evaluate the therapeutic potential of our recently developed *Salmonella*-based vaccination strategy [27]. We decided to apply four different immunization strategies (A–D) to BALB/c mice (Fig. 1a–d). Animals of all immunization groups received the fibrosarcoma WEHI-p60 subcutaneously on day 0. Tumor growth was measured between days 4 and 14. Mice of the immunization group A obtained an intravenous vaccination with SB824 (pHR241) on day 0 simultaneously with tumor injection (Fig. 1a, black triangle). Control mice of the immunization group S were immunized with plasmidless SB824 (Fig. 1a, black diamond) or remained non-immunized (NI) (Fig. 1a, black square). In Fig. 1a, it is demonstrated that in mice vaccinated with SB824 (pHR241) peak fibrosarcoma growth on day 7 (35 ± 6 mm^2) was significantly reduced on day 10 (18 ± 6 mm^2) and on day 14 (9 ± 5 mm^2) after tumor injection. Interestingly, mice of the immunization group S revealed a transient tumor growth delay during the first days after vaccination. However, immunization with SB824 did not result in the reduction of WEHI-p60 growth on day 14 (63 ± 7 mm^2) as compared to non-immunized mice (63 ± 9 mm^2).

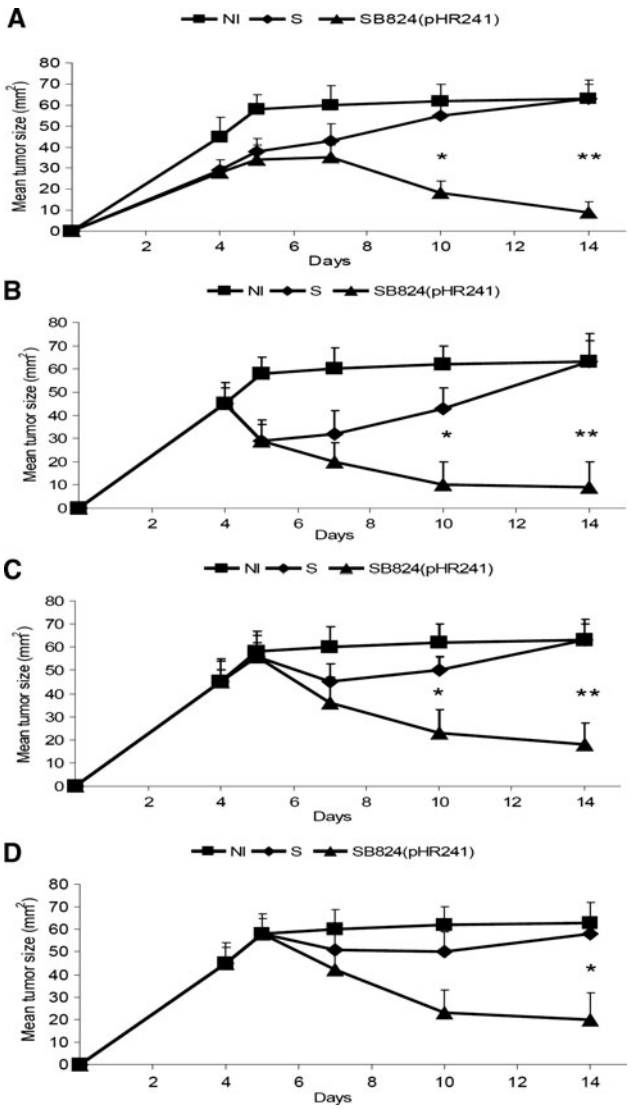

Fig. 1 Regression of fibrosarcoma growth after therapeutic vaccination with recombinant *Salmonella*. BALB/c mice received a subcutaneous injection of 5 × 10^6 WEHI-p60 cells into the flank on day 0. Mice given the fibrosarcoma cells were inspected on days 4, 5, 7, 10 and 14 for tumor growth and size. **a** Immunization group A: application of *Salmonella* vaccine strain SB824 (pHR241) expressing translocated YopE/p60 via the intravenous route on day 0. **b** Immunization group B: application of *Salmonella* vaccine strain SB824 (pHR241) via the intravenous route on day 4. **c** Immunization group C: application of *Salmonella* vaccine strain SB824 (pHR241) via the orogastric route on day 0. **d** Immunization group D: application of *Salmonella* vaccine strain SB824 (pHR241) via the orogastric route on day 4. Negative control mice were immunized with plasmidless SB824 (*S*) or remained non-immunized (*NI*). Means and standard deviations of ten mice per vaccination group are indicated. *Asterisks* indicate values that differ significantly from the corresponding values obtained from mice of the control groups S and NI ($*p < 0.01$, $**p < 0.005$)

Mice of immunization group B received an intravenous vaccination with SB824 (pHR241) 4 days after tumor injection (Fig. 1b, black triangle). Control mice of immunization group S were immunized with plasmidless SB824 (Fig. 1b, black diamond) or remained non-immunized (NI) (Fig. 1b, black square). In Fig. 1b, it is shown that in mice vaccinated with SB824 (pHR241) peak fibrosarcoma growth on day 4 (45 ± 7 mm^2) gradually declined on day 5 (29 ± 9 mm^2) and day 7 (20 ± 8 mm^2), and was significantly reduced on day 10 (10 ± 10 mm^2) and on day 14 (9 ± 11 mm^2). Interestingly, mice of the immunization group S revealed a transient tumor growth delay during the first days after vaccination. However, immunization with SB824 did not result in the reduction of WEHI-p60 growth on day 14 (63 ± 12 mm^2) as compared to non-immunized mice (63 ± 9 mm^2).

Mice of immunization groups C and D were orogastrically vaccinated with recombinant *Salmonella*. Animals of the immunization group C obtained vaccination with SB824 (pHR241) on day 0 simultaneously with tumor injection (Fig. 1c, black triangle). Control mice of the immunization group S were immunized with plasmidless SB824 (Fig. 1c, black diamond) or remained non-immunized (NI) (Fig. 1c, black square). Figure 1c reveals that in mice vaccinated with SB824 (pHR241), peak fibrosarcoma growth on day 5 (56 ± 11 mm^2) was significantly reduced on day 10 (23 ± 10 mm^2) and on day 14 (18 ± 9 mm^2) after tumor injection. Interestingly, mice of the immunization group S revealed a transient tumor growth delay during the first few days after vaccination. However, immunization with SB824 did not result in the reduction of WEHI-p60 growth on day 14 (63 ± 14 mm^2) as compared to non-immunized mice (63 ± 9 mm^2).

Mice of the immunization group D received an orogastric vaccination with SB824 (pHR241) 4 days after tumor injection (Fig. 1d, black triangle). Control mice of immunization group S were immunized with plasmidless SB824 (Fig. 1d, black diamond) or remained non-immunized (NI) (Fig. 1d, black square). In Fig. 1d, it is demonstrated that in mice vaccinated with SB824 (pHR241) peak fibrosarcoma growth on day 5 (58 ± 9 mm^2) gradually declined on day 7 (42 ± 9 mm^2) and day 10 (23 ± 10 mm^2), and was significantly reduced on day 14 (20 ± 12 mm^2). Interestingly, mice of immunization group S revealed a transient tumor growth delay during the first few days after vaccination. However, immunization with SB824 did not result in the reduction of WEHI-p60 growth on day 14 (58 ± 7 mm^2) as compared to non-immunized mice (63 ± 9 mm^2).

Taken together, all four immunization strategies (A–D) using SB824 (pHR241) as a vaccine strain resulted in a highly significant reduction of fibrosarcoma size as compared to control mice.

Determination of the rate of tumor-free mice after intravenous or orogastric vaccination with recombinant *Salmonella*

In our previous study, we demonstrated in a prophylactic anti-tumor immunization setting that 80% of all tumor-challenged mice remained free of fibrosarcoma growth [27]. Gray bars in Fig. 2 reveal the percentages of tumor-free mice after intravenous or orogastric therapeutic vaccination with recombinant *Salmonella.* Determination of tumor growth was performed 14 days after WEHI-p60 inoculation. All mice from immunization groups A–D vaccinated with SB824 (pHR241) showed significantly higher tumor regression rates as compared to mice vaccinated with SB824 (immunization group S, $5 \pm 4\%$) or to mice that remained non-immunized (immunization group NI, $4 \pm 2\%$). While $71 \pm 4\%$ of mice from the immunization group A showed a complete regression of tumor growth, mice from immunization group B revealed an even higher regression rate ($80 \pm 2\%$). In contrast to intravenous vaccination with *Salmonella*, orogastric immunization led to significantly lower ($^{**}p < 0.005$) protection rates (immunization group C, $52 \pm 4\%$; immunization group D, $50 \pm 5\%$). No significant differences could be detected between immunization groups A and B or C and D, respectively.

Co-staining of $p60_{217–225}$-specific CD8 T cells with CD62L and CD127

We assumed that regression of fibrosarcoma growth was mediated by $p60_{217–225}$-specific CD8 induced by vaccination with SB824 (pHR241). Antigen-specific T cells can be divided at least into three subsets [36]. Effector CD8 T cells (TEC) are characterized by an immediate effector function, poor proliferative activity and a limited in vivo survival. In contrast, memory T cells persist for extended periods due to an antigen-independent homeostatic turnover. Central memory T cells (TCMC) reside preferentially in lymphoid organs and lack immediate effector functions [20], whereas effector memory T cells (TEMC) migrate mainly into non-lymphoid tissue and elicit immediate effector functions on antigen reencounter. Recent results indicate that interleukin-7 receptor α-chain (CD127) surface expression is a marker for long-living memory T cells [15]. The combination of surface staining for CD127 and L-selectin (CD62L) further separates between TCMC ($CD127^{high}/CD62L^{high}$) and TEMC ($CD127^{high}/CD62L^{low}$) allowing to distinguish TEC ($CD127^{low}/CD62L^{low}$) from memory T cells at early time points of in vivo immune responses.

To demonstrate that both long- and short-living CD8 T cells with an immediate effector function are induced by

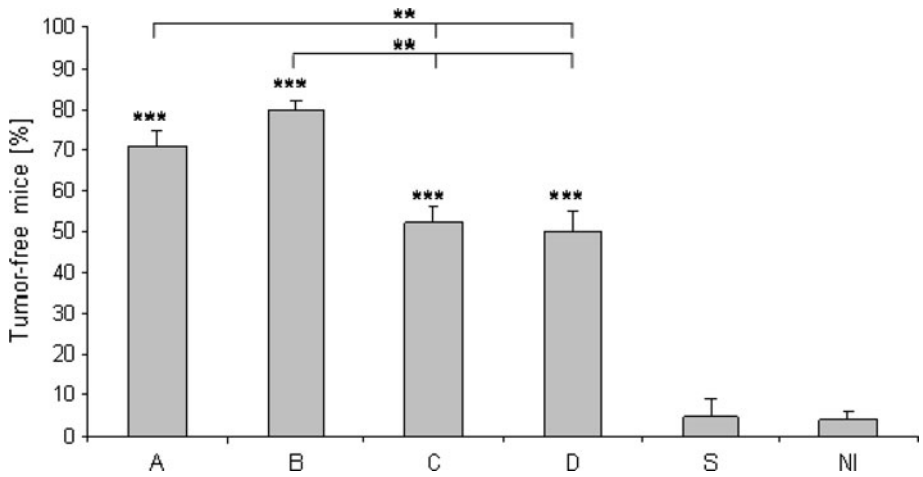

Fig. 2 Percentages of tumor-free mice after intravenous or orogastric therapeutic vaccination with recombinant *Salmonella*. Determination of tumor size was performed 14 days after WEHI-p60 inoculation. Complete tumor regression was defined by a calliper detection threshold of 3 mm × 3 mm, successful protection against tumor rechallenge after 1 month and no cancer relapse during the observation period of 3 months. Immunization group A: application of *Salmonella* vaccine strain SB824 (pHR241) via the intravenous route on day 0. Immunization group B: application of SB824 (pHR241) via the intravenous route on day 4. Immunization group C: application of SB824 (pHR241) via the orogastric route on day 0. Immunization group D: application of SB824 (pHR241) via the orogastric route on day 4. Negative control mice were immunized with plasmidless SB824 (*S*) or remained non-immunized (*NI*). Means and standard deviations of ten mice per vaccination group are indicated. *Asterisks* indicate values that differ significantly from the corresponding values obtained from mice of control groups *S* and *NI* (***$p < 0.001$) or indicate values of groups A and B that differ significantly from those of groups C and D (**$p < 0.005$)

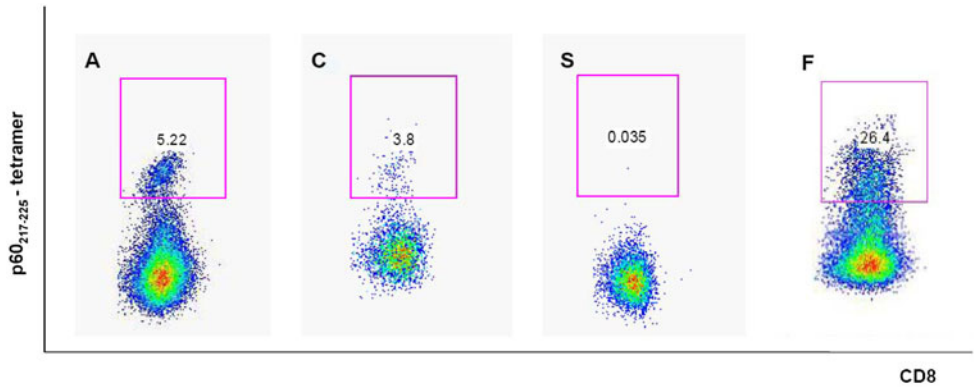

Fig. 3 Representative dot plots of $p60_{217–225}$-tetramer-positive CD8 T cells in blood samples (*panels A*, *C*, and *S*) or tumor tissue (*panel F*) from BALB/c mice ($n = 5$ per group) determined 2 weeks after WEHI-p60 fibrosarcoma inoculation. *Panels A* and *F* show representative *dot plots* of mice immunized intravenously with SB824 (pHR241); *panel C* shows a representative dot plot of mice vaccinated with SB824 (pHR241) via the orogastric route. *Panel S* depicts a representative *dot plot* of mice vaccinated intravenously with plasmidless SB824

our therapeutic immunization strategies using recombinant *Salmonella*, blood and tumor samples from immunized mice were collected 14 days after tumor injection and $p60_{217–225}$ tetramer-positive CD8 T cells were analyzed by flow cytometry (Figs. 3, 4). Our data show that p60-specific CD8 T cells could barely be detected in blood samples of mice vaccinated with plasmidless SB824 (Fig. 3, panel S). In contrast, intravenous or orogastric immunization with SB824 (pHR241) expressing translocated YopE/p60 resulted in a pronounced stimulation of $p60_{217–225}$ tetramer-positive CD8 T cells in blood samples (Fig. 3, panels A and C) or fibrosarcoma tissue (Fig. 3, panel F). Furthermore, Fig. 5 reveals that vaccination with SB824 (pHR241) (immunization groups A–D) induces significantly higher absolute numbers of p60-specific CD8 T cells (500–2,000 per ml blood) as compared to immunization with plasmidless SB824 (38 ± 17 p60-specific CD8 T cells/ml blood, S) or non-immunized mice (42 ± 9 p60-specific CD8 T cells/ml blood, NI). The comparison of antigen-specific CD8 T cell responses in mice after intravenous vaccination (Fig. 5a, b) with mice after orogastric immunization (Fig. 5c, d) demonstrates that significantly

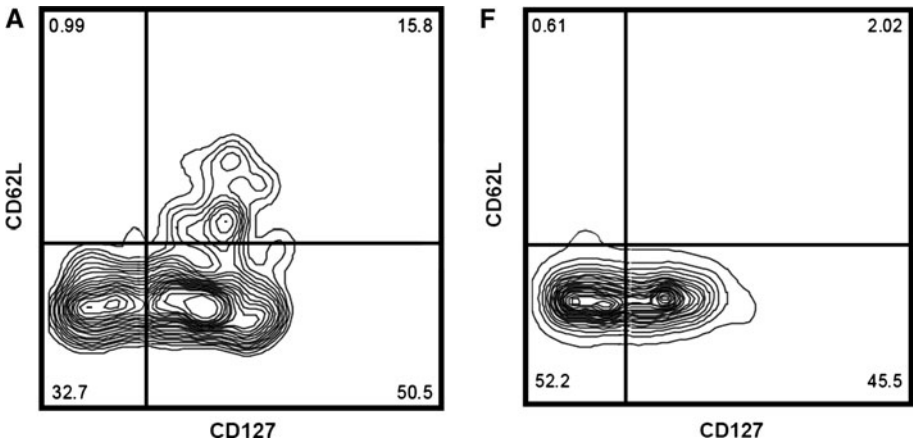

Fig. 4 Differentiation patterns of p60-specific CD8 T cells into phenotypically distinct subsets in blood samples (*panel A*) or tumor tissue (*panel F*) from BALB/c mice ($n = 5$ per group) determined 2 weeks after WEHI-p60 fibrosarcoma inoculation and intravenous vaccination with SB824 (pHR241). Cells were stained for expression of CD127 (*x*-axis) and CD62L (*y*-axis). The percentage of cells in each quadrant is indicated. $CD127^{high}/CD62L^{high}$ central memory CD8 T cells (TCMC); $CD127^{high}/CD62L^{low}$ effector memory CD8 T cells (TEMC); $CD127^{low}/CD62L^{low}$ effector CD8 T cells (TEC)

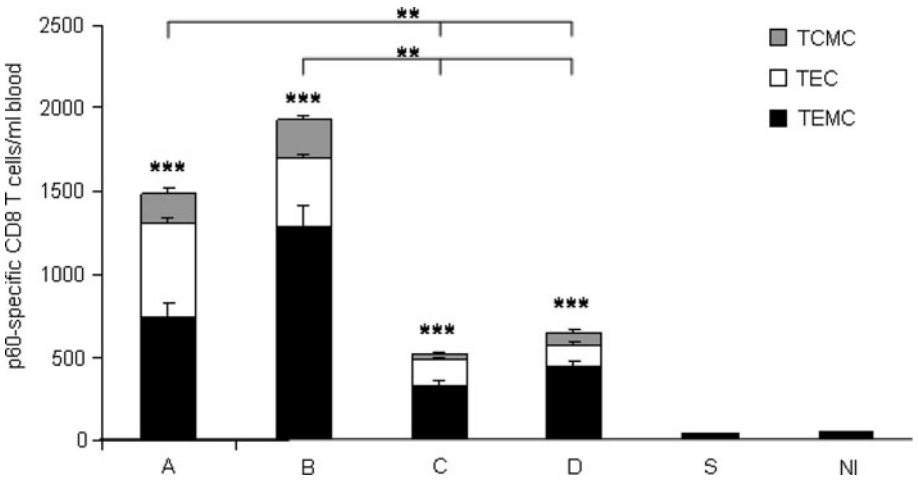

Fig. 5 Absolute numbers of p60-specific CD8 T cells per milliliter blood and differentiation patterns of phenotypically distinct subsets (TEMC, TEC and TCMC) on day 14 after tumor inoculation. Cells were stained for expression of CD127 and CD62L. Immunization group A: application of *Salmonella* vaccine strain SB824 (pHR241) via the intravenous route on day 0. Immunization group B: application of SB824 (pHR241) via the intravenous route on day 4. Immunization group C: application of SB824 (pHR241) via the orogastric route on day 0. Immunization group D: application of SB824 (pHR241) via the orogastric route on day 4. Immunization group S: application of SB824 via the intravenous route on day 0. *NI*: non-immunized mice received phosphate-buffered saline on day 0. *Asterisks* indicate values that differ significantly from the corresponding values obtained from mice of the control groups *S* and *NI* (***$p < 0{,}001$) or indicate values of groups A and B that differ significantly from those of group C and D (**$p < 0.005$)

higher absolute numbers of $p60_{217–225}$ tetramer-positive CD8 T cells were found in blood samples from mice of immunization groups A and B ($1{,}487 \pm 176$ and $1{,}928 \pm 179$ p60-specific CD8 T cells/ml blood) than in mice from groups C and D (524 ± 65 and 652 ± 63 p60-specific CD8 T cells/ml blood). These results are in line with our above described observations (Fig. 2) that intravenous vaccination with *Salmonella* leads to significant higher tumor regression rates than orogastric vaccination. Further analysis of lymphocytes from blood samples revealed a predominance of CD8 T cells with a $CD62L^{low}$-phenotype (Fig. 5). Thus, both TEMC and TEC constitute the majority of p60-specific lymphocytes in this non-lymphoid compartment, whereas relatively low levels of TCMC were detected.

Detection of p60-specific CD8 T cells in fibrosarcoma tissue

Immune cells such as antigen-specific CD8 T cells enter the skin and the subcutaneous compartment via the blood stream. As shown above, we analyzed the distribution of

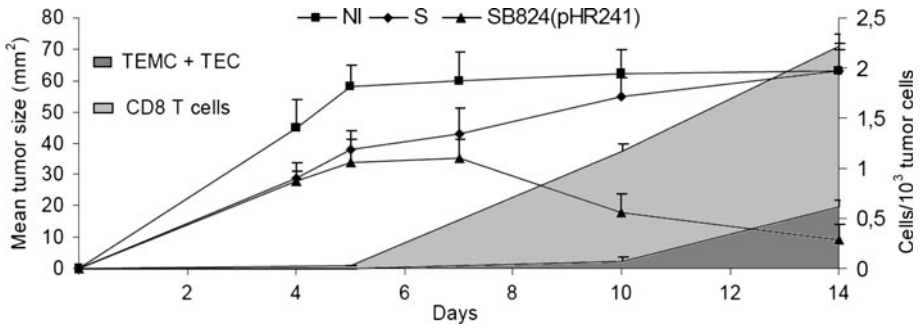

Fig. 6 Detection of tumor-invading p60-specific CD8 T cells in fibrosarcoma tissue. Regression of tumor growth in mice intravenously vaccinated on day 0 with SB824 (pHR241) (see Fig. 1a) was plotted against the numbers of CD8 T cells (*light gray area*) and $p60_{217-225}$ tetramer-positive TEC and TEMC (*dark gray area*), respectively, per 10^3 tumor cells. Means and standard deviations of ten mice per vaccination group are indicated

TEC, TCMC and TEMC subpopulations in peripheral blood (Fig. 5). We found that the majority of p60-specific lymphocytes belonged to the TEC and the TEMC CD8 T cell subsets. As recently demonstrated, WEHI-p60 cells are recognized in vitro by p60-specific CD8 T cells [27], so we hypothesized this to happen in vivo as well. Most likely, TEC and TEMC are responsible for the in vivo destruction and regression of inoculated WEHI-p60 cancer cells in our tumor model. To determine whether tumor regression was associated with the presence of p60-specific TEC and TEMC, fibrosarcoma tissue of mice from immunization group A underwent FACS analysis and tetramer staining. As shown in Fig. 6, we plotted WEHI-p60 growth against the numbers of CD8 T cells (light gray area) and $p60_{217-225}$ tetramer-positive TEC and TEMC (dark gray area), respectively, per 10^3 tumor cells. Interestingly, CD8 T cells are already detectable in the tumor tissue on day 5 ($0.026 \pm 0/10^3$ tumor cells). At this time point, no p60-specific CD8 T cells could be detected. However, on day 10 after fibrosarcoma challenge, $p60_{217-225}$ tetramer-positive TEC and TEMC ($0.0648 \pm 0.046/10^3$ tumor cells) were found in cancer tissue culminating 4 days later in a tenfold higher concentration ($0.612 \pm 0.072/10^3$ tumor cells).

In summary, these results strongly support the hypothesis that tumor-invading p60-specific effector T cells contribute to the destruction of WEHI-p60 cells and the regression of this solid tumor.

Discussion

This study demonstrates that recombinant *Salmonella* can lead to a pronounced antigen-specific effector CD8 T cell induction, resulting in tumor regression in mice therapeutically immunized via the intravenous or the orogastric route. It is also shown that these effector lymphocytes accumulate in the regressing tumor tissue. Furthermore, we found that intravenous immunization induces significantly higher antigen-specific CD8 T cell frequencies and tumor regression rates as compared to the orogastric immunization route.

Anti-tumor vaccines are able to successfully promote tumor-specific T cell responses in both preclinical and clinical studies [25, 38]. However, induction of antitumor T cell immunity rarely results in an effective eradication of established diseases in murine models or patients [31]. It is well-established that CD8 T cells play a central role in the host defense against tumors [14]. However, the induction of antigen-specific effector CD8 T cell responses to fight cancer remains a major challenge in vaccine development. Live attenuated bacteria (e.g., *Salmonella enterica*, *Listeria monocytogenes* and *Mycobacteria*) have been widely used as vaccine carriers for foreign antigen display to induce cell-mediated immunity [7, 17]. In the past, the T3SS of *S. enterica* serovar Typhimurium became an attractive tool for heterologous protein delivery directly into the cytosol of macrophages and dendritic cells resulting in vigorous antigen-specific CD8 T cell priming and the induction of protective immunity against viruses, bacteria and tumors [27, 32, 34, 40]. Previously, we have demonstrated that a T3SS effector molecule from *Yersinia* can be used as a carrier protein for translocation of the large *Listeria*-derived p60 antigen by *Salmonella*'s T3SS [33]. After a single oral immunization of mice with recombinant *Salmonella* expressing translocated YopE/p60, the efficient stimulation of p60 peptide-specific CD8 T cells led to protection against listeriosis. Using this model for prophylactic anti-cancer therapy, we demonstrated that 80% of all *Salmonella*-vaccinated mice were protected against a subcutaneous challenge with a progressively grown fibrosarcoma transfected with p60. Furthermore, the remaining 20% of mice showed significant reduction in the development of tumor size as compared to control groups [27].

Based on these findings, we became interested in the model's potential for therapeutic anti-cancer intervention.

The idea of using bacteria as an anti-cancer treatment is quite old. William Coley described already in 1893 a transient regression of solid, especially soft tissue tumors after treatment with Gram-negative bacteria [5]. Today, we know that the lipopolysaccharides on the *Salmonella* surface enable CD14+ monocytes to secrete TNF, IL-1 and IL-6, which could explain the temporary tumor regression or growth delay during the first few days after *Salmonella* vaccination in our vaccination setting [30, 42]. Except or Bacille Calmette-Guérin (BCG) instillation in the treatment of superficial bladder cancer [24], to date the use of bacteria in anti-cancer therapy has shown limited success.

In the majority of tumor vaccination studies, post-therapy antitumor activity is assessed by monitoring peripheral T cell immunity [40]. This strategy provides a convenient and accurate method for quantification of tumor-specific T cells. However, it does not predict whether these cells will maintain effector activity once they encounter the highly immune-suppressive tumor milieu characterized by inhibitory cytokines, enzymes, death receptor ligands and T suppressor cells [23, 35, 41]. Probably, one of the best documented and most crucial mechanisms of cancer escape is the total or partial loss of MHC class I molecules in tumor cells, resulting in an insufficient presentation of antigenic peptides to the immune system [9, 10]. Further studies are needed to investigate whether genetically manipulated *Salmonella* vaccine strains can influence the immune-suppressive microenvironment of tumors.

Mainly, three criteria are required for destruction of tumor cells by the immune system [18]: first of all, sufficient numbers of naïve immune cells with highly avid recognition of tumor antigens must be generated in vivo; secondly, these antigen-specific cells must proliferate, survive and migrate to the tumor; and thirdly, especially in the case of solid tumors, they must infiltrate the tumor tissue to destroy the cancer cells efficiently. What subpopulation of tetramer-positive $p60_{217–225}$-specific CD8 T cells conferred protection against WEHI-p60 fibrosarcoma cells in our experimental setup? It has been demonstrated that in the murine *Listeria* infection model, TEMC are the most effective CD8 T cell subsets conferring protection against this intracellular bacterium [1, 16]. Furthermore, the relationship between tumor-infiltrating T cells and improved clinical outcome and survival has been shown in various studies [4, 6, 26, 29, 43]. Since $p60_{217–225}$-tetramer-positive TEC and TEMC represented the majority of p60-specific CD8 T cells in the peripheral blood of mice from all immunization groups vaccinated with SB824 (pHR241), it was tempting to speculate that these lymphocyte subsets were also present in the fibrosarcoma tissue. Even though it remains unclear whether a more rapid or a more effective induction of effector CD8 T cells caused the more successful outcome of the intravenous immunization route, a clearly positive correlation ($\tau = 0.666$) between the amount of effector T cells in blood (Fig. 5) and the probability of tumor regression (Fig. 2) could be proven. Additionally, a pronounced accumulation of antigen-specific effector T cells was detected in the fibrosarcoma tissue (Figs. 3f, 4f) representing up to a third of all tumor-invading CD8 T cells correlating with the kinetics of tumor regression (Fig. 6).

The data presented in this report clearly demonstrate that our vaccination approach using T3SS-mediated antigen delivery by *Salmonella* is a very promising tool for future anti-cancer therapies. Recombinant *Salmonella* is able to induce sufficient amounts of p60-specific CD8 T cells in blood. These lymphocytes can migrate to the tumor, invade the tumor tissue and, according to the observed tumor regression and former in vitro experiments [27], most likely destroy the tumor cells. Further studies are needed to determine whether this vaccination approach can also be successful against cancer cells expressing naturally occurring tumor-associated antigens. It will be exciting to investigate if *Salmonella* may fulfill the promise suggested by the data presented in this proof-of-principle report.

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