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ORIGINAL ARTICLE

Cancer immunotherapy based on recombinant *Salmonella enterica* serovar Typhimurium *aroA* strains secreting prostate-specific antigen and cholera toxin subunit B

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Prostate cancer is the most common malignant tumor in men and is normally associated with increased serum levels of prostate-specific antigen (PSA). Therefore, PSA is one potential target for a prostate cancer vaccine. In this study we analyzed the functionality of new bacterial PSA vaccines, expressed and secreted via the hemolysin (HlyA) secretion system of *Escherichia coli*, the prototype of Type I secretion systems (T1SS) using an attenuated *Salmonella enterica* serovar Typhimurium *aroA* strain as carrier. The data demonstrate that a bacterial live vaccine encompassing T1SS in combination with cholera toxin subunit B can be successfully used for delivery of PSA to induce cytotoxic $CD8^+$ T-cell responses resulting in an efficient prevention of tumor growth in mice.

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Keywords: bacterial cancer vaccines; prostate-specific antigen; cholera toxin subunit B; hemolysin (HlyA) secretion system (T1SS); cancer immunotherapy

Introduction

Prostate cancer is the most common form of cancer affecting men in the United States, and is the second leading cause of cancer deaths among men each year.[1] These facts emphasize the need for new therapeutic approaches against prostate cancer. One of the promising new strategies in this field is an immunotherapeutic approach based on prostate-related antigens such as prostate-specific antigen (PSA). This protein is a 34 kDa glycoprotein, primarily produced by the prostate ductal and acinar epithelium.[2] In cancer cells the expression of PSA is markedly increased compared to normal prostatic cells.[3] This unique expression pattern explains why PSA might be an excellent antigenic target for immunotherapy. Several PSA vaccines consisting of dendritic cell vaccines[4,5] PSA-derived peptide[6] recombinant vaccinia virus[7] and recombinant DNA vaccines[8,9] are currently under investigation in clinical trials.

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This study describes a new strategy for achieving an anti-tumor immune response against PSA using an attenuated *Salmonella enterica* serovar Typhimurium *aroA* strain as carrier. In the past, several groups have shown that *Salmonella* strains alone or used as a carrier for cancer vaccines can provide protection against melanoma,[10] renal carcinoma,[11] breast cancer,[12] non-small cell lung carcinomas[13] and prostate cancer[14] in mice. However, until now, there are no licensed bacterial vaccines for human cancer therapy.

To develop a PSA vaccine for human immunotherapy we generated a new plasmid system, allowing the expression and the secretion of the cancer antigen together with cholera toxin subunit B (CtxB). The native CtxB is one of the best studied and most effective experimental mucosal adjuvants known today.[15,16] In addition, this protein is a highly efficient carrier molecule for chemically or genetically conjugated antigens for eliciting mucosal and systemic antibody responses, as well as mucosal tolerance for prophylactic vaccines against autoimmune diseases.[17-19] To test the hypothesis that CtxB can enhance the antitumor efficacy of a cancer vaccine, several plasmids, encoding different combinations between PSA and CtxB, were generated. In all cases PSA was delivered as a hemolysin fusion via the hemolysin (HlyA) secretion system of *Escherichia coli*, which allows efficient protein secretion from the

Salmonella carrier.[20] In this context, several groups have demonstrated that the HlyA secretion machinery can not only be used in the classical field of anti-infective vaccination[21–23] but also as delivery system for immunocontraceptive vaccines,[24] for coexpression and co-delivery of active cytokines,[25] and for cancer vaccines.[26]

In this study we have shown that a CtxB–PSA fusion can induce a more efficient PSA-specific response compared to PSA alone, when secreted via the hemolysin pathway. Furthermore, oral immunization of mice against *Salmonella*, secreting this CtxB–PSA fusion protein, resulted in protection against tumor cell lines recombinantly expressing PSA *in vivo*.

Materials and methods

Bacterial strains, plasmids, cell lines and mice

The bacterial strains, plasmids, cell lines and mice used in this study are listed in Table 1.

The bacteria were grown in either Luria–Bertani (LB; Sigma, Germany), or brain heart infusion (BHI; BD, Germany), medium with appropriate antibiotics (Sigma). For selection $100\,\mu g\,ml^{-1}$ ampicillin and $25\,\mu g\,ml^{-1}$ kanamycin were used for all strains

Construction of pcDNA3-PSA

For the generation of pcDNA3-PSA, pBS/PSA1, provided by John G Frelinger,[27] was digested with *Sal*I/*Bam*HI, and subcloned into the corresponding *Sal*I/*Bam*HI vector fragment of pUC18. Subsequently, the resulting vector pUC18 PSA was cut with *Hin*dIII, and the PSA-containing fragment was ligated into the *Hin*dIII-digested eukaryotic expression vector pcDNA3 (Invitrogen, Germany). PcDNA3-PSA was sequenced and used for the generation of cell lines and immunization of mice. For immunization experiments, plasmid DNA was purified, using the Qiagen Endotoxin Free Plasmid Purification kit (Qiagen, Hilden, Germany), and preserved

Table 1 Bacterial strains, plasmids, cell lines and mice

Name	*Relevant characteristics/sequence*	*Source or reference*
Bacterial strains		
E. coli DH5α	F^-, ϕ80d*lacZ*ΔM15, Δ(*lacZYA-argF*)U169 *deoR*, *recA*1, *endA*1, *hsdR*17(rk^-, mk^+), *phoA*, *supE*44, λ^-, *thi*-1, *gyrA*96, *relA*1	Invitrogen
E. coli BW25141	(*lacI*^{q} *rrnB*$_{T14}$ Δ*lacZ*$_{WJ16}$ Δ*phoBR580 hsdR514* Δ*araBAD*$_{AH33}$ Δ*rhaBAD*$_{LD78}$ *galU95* *endA*$_{BT333}$ *uidA*(Δ*MluI*)::*pir*$^+$ *recA1*)	Datsenko and Wanner[28]
Salmonella enterica serovar Typhimurium *aroA* SL7207	*his*G46, DEL407 (*aroA*544::Tn10 (Tc^s))	B.A.D. Stocker
Plasmids		
pcDNA3	pCMV, ampicillin resistance (Ap^R), neomycin, SV 40, ColE	Invitrogen
pcDNA3-PSA	*psa* cDNA in pcDNA3	This study
pGEX-6p-1-Plasmid	Ap^R, tag-promoter	Amersham Bioscience
pKD4	kanamycin resistance (Kan^R)	Datsenko and Wanner[28]
pKD46	Ap^R, encoding the Red recombinase Expresses γ, β and *exo* from the arabinose-inducible P_{araB} promoter	Datsenko and Wanner[28]
DNA of *V. cholerae* El tor		J Reidl
pMOhly1	Ap^R, *hlyR, hlyC, hlyAs* (encoding the hemolysin secretion signal), *hlyB, hlyD*	Gentschev *et al.*[20]
pMKhly1	Kan^R-derivate of pMOhly1	This study
pMKhly-CtxB	Kan^R, *hlyR, hlyC, ctxB-hlyAs* (encoding a cholera toxin subunit B (CtxB)–HlyAs fusion), *hlyB, hlyD*	This study
pMKhly-PSA	Kan^R, derivate of pMKhly1 encoding a prostate-specific antigen (PSA)–HlyAs fusion	This study
pMKhly-CtxB-PSA	Kan^R, derivate of pMKhly-CtxB, encoding a CtxB–PSA–HlyAs fusion	This study
pMOhly-Psa/CtxB	Kan^R, derivate of pMKhly-PSA carrying the *ctxB* gene under the control the Tag promoter	This study
Cell lines		
P815	Mouse lymphoblast-like mastocytoma cell line	ATCC (Rockville, MD, USA)
PPSA 18	pcDNA-PSA transfected P815 cells	This study
Mice		
DBA/2	Wild-type (H-2^d)	Harlan-Winkelmann, Borche, Germany

until use at a concentration of 1 μg ml^{-1} in endotoxin-free phosphate-buffered saline (PBS) at −20 °C.

Construction of kanamycin-resistant pMOhly1 derivate
The replacement of the ampicillin conferring resistance cassette of pMOhly1 was performed basically as described before.[28] Briefly, the sense primer P1:
5′-**GAGTATTCAACATTTCCGTGTCGCCCTTATTC CCTTTTTTG**GTGTAGGCTGGAGCTGCTTC-3′, the antisense primer P2:
5′-**GCGATCTGTCTATTTCGTTCATCCATAGTTGC CTGACTCCC**CATATGAATATCCTCCTTA-3′ and the plasmid pKD4 as template were used for PCR to produce a fragment carrying the kanamycin-resistant gene (Kan^R) flanked by regions homologous to the ampicillin-resistant gene (bold). *E. coli* strain BW25114, harboring the pKD46 plasmid and the target plasmid pMOhly1, was grown in LB medium supplemented with 0.2% L-(+)-arabinose for 3–4 h at 37 °C before the PCR fragment was being transformed. After the transformation, the bacterial cells were spread on LB agar plates containing 25 μg ml^{-1} kanamycin and left at 37 °C overnight. The next day, Kan^R clones were picked and incubated for additional 48 h in LB medium, containing 50 μg ml^{-1} kanamycin, to get rid of all ampicillin resistance-conferring plasmids. Finally, clones with a Kan^R and ampicillin-sensitive phenotype were selected. The replacement of the Ap^R gene by the Kan^R cassette was confirmed by PCR and sequencing. The resulting plasmid was called pMKhly1.

Cloning of pMKhly-PSA plasmid
The sense primer PSA-Nsi1: 5′-GATTGGTGATGCAT CCCTCAT-3′ (*Nsi*I restriction sites are underlined), the antisense primer PSA-Nsi2: 5′-GGTGCTCATGCAT TGGCCACG-3′ and the plasmid pcDNA-PSA as template were used in PCR to amplify a DNA fragment encoding PSA without the N-terminal signal. PCRs were performed in a Thermal Cycler 60 (Biometra, Göttingen, Germany): 30 cycles of 1 min at 94 °C, 1 min at 54 °C and 2 min at 72 °C.

After digestion with the *Nsi*I restriction enzyme the DNA fragment, carrying the *psa* gene, was inserted into the single *Nsi*I site of the export vector pMKhly1. The resulting plasmid pMKhly-PSA was isolated from *E. coli* DH5α (Invitrogen). It was then assayed by restriction analysis and sequenced.

Cloning of pMKhly-PSA/CtxB plasmid
The sense primer Ptac-*Sal*I 5′-AAAAAAGTCGACGG CTGTGCAGGTCGTAAATCACTGC and the antisense primer Ptac-*Not*I 5′-AAAAAAGCGGCCGCGAAATT GTTATCCGCTCACAATTCC were used for the PCR amplification of a 201 bp DNA fragment encoding the Ptac-Promotor from pGEX-6p-1 plasmid (Amersham Bioscience, Germany). The PCR was performed in a T3 Thermocycler (Biometra): 30 cycles of 30 s at 95 °C, 30 s at 55 °C and 90 s at 72 °C. The sense primer Rbs-*Not*I-forward 5′-AAAAAAGCGGCCGCTAAGGATGAAT TATGATTAAATTAAAATTTGG and the antisense primer ctb-*Sal*I-reverse 5′-TTTATAGTCGACTTAATT TGCCATACTAATTGCGGCAATCGC were used for the PCR amplification of the 413 bp DNA-fragment encoding the ribosome-binding site and the whole coding sequence of *ctxB* from *Vibrio cholerae* El tor. The PCR was performed in a T3 Thermocycler (Biometra): step1: 30 cycles of 30 s at 95 °C, 30 s at 50 °C and 2 min at 72 °C. After purification with the QIAquick PCR Purification Kit (Qiagen) and digestion of both fragments with the *Not*I restriction enzyme, the two fragments were ligated, resulting in the 594 bp Ptac-*ctx*B fragment. The resulting fragment was also purified and, after it had been digested with the *Sal*I restriction enzyme, was inserted into the single *Sal*I site of the export vector pMKhly-PSA. The resulting plasmid pMKhly-PSA/CtxB was isolated from *E. coli* DH5α (Invitrogen, Karlsruhe, Germany), analyzed and sequenced.

Cloning of pMKhly-CtxB-PSA plasmid
A chromosomal DNA of the *V. cholerae* El tor strain (Table 1), the sense primer 5′*ctxB Nsi*I GCATATGC ACATGCATCACCTCAAAATATTACTGAT and the antisense primer 3′ *ctxB Srf*I *Nsi*I GGCTTTTTTATATC TTATGCAT**GCCCGGGC**ATTGCGGCAATCGC (*Srf*I site is bold) were used for the PCR amplification of an ~300 bp DNA fragment representing the *ctxB* gene without the original leader sequence. After purification with the QIAquick PCR Purification Kit (Qiagen) and digestion with the *Nsi*I restriction enzyme, the DNA fragment, carrying the whole *ctxB* gene without the N-terminal signal sequence, was inserted into the single *Nsi*I site of the export vector pMKhly1 (Table 1). The resulting plasmid pMKhly-CtxB was isolated from *E. coli* DH5α (Life Technologies, Karlsruhe, Germany), analyzed and sequenced.

The human *psa* gene was amplified from the pCDNA3-PSA plasmid by PCR using 5-PSA-Blunt GTGGGAGG CTGGGAGTGC and 3-PSA-Blunt GGGGTTGGCCAC GATGGT primers. The PCR was carried out in a Thermal Cycler 60 (Biometra) for 30 cycles of 1 min at 94 °C, 1 min at 56 °C and 2 min at 72 °C. The 0.7 kb DNA product was subsequently cloned into the *Srf*I site of pMKhly-CtxB. The resulting pMKhly-CtxB-PSA plasmid was examined by restriction analysis and sequencing.

Construction of the PSA-transfected cell line PPSA 24
The cell line P815 PSA clone 18 (PPSA 18) was generated by stably transfecting P815 with pcDNA3-PSA. For further use, the PPSA 18 was chosen. The expression of PSA was detected in western blot analysis which remained stable for at least 1 month in culture (data not shown).

SDS–PAGE and western blot
Bacteria were grown in BHI medium to a density of 1×10^9 cells per ml. About 20 ml of the culture was centrifuged for 30′ at 4000 r.p.m. and 4 °C in a Hereaus centrifuge. About 18 ml of the supernatant was transferred into a fresh tube. Subsequently, 1.8 ml trichloracetic acid (Applichem, Darmstadt, Germany) was added; the liquid was mixed and incubated on ice for at least 1 h. After incubation, the suspension was centrifuged for

30′ at 4000 r.p.m. and 4 °C in a Hereaus centrifuge. The supernatant was decanted and the pellet was washed with 1 ml aceton p.a. (Applichem); the precipitate was centrifuged for 10′ at 4000 r.p.m. and 4 °C in a Hereaus centrifuge. The pellet was air-dried and taken up in 150 µl 5 × Laemmli buffer with or without β-mercaptoethanol.[29] About 20 µl of the solution was used for each lane in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE). The separated proteins were electrophoretically transferred to Hybond ECL nitrocellulose membrane (Amersham Pharmacia, Little Chalfont, UK) and blocked overnight with PBS containing 1% bovine serum albumin (BSA). The membrane was washed in PBS–Tween 0.05%, incubated with polyclonal rabbit anti-PSA antibody (1:750, Dako, Denmark), CtxB antibody (1:1000, Zytomed, Berlin, Germany) or HlyAs antibody[20] and subsequently incubated with horseradish peroxidase-coupled anti-rabbit immunoglobulin G (1/2000; Dianova, Hamburg, Germany) for 1 h. The western blot was carried out using the enhanced chemiluminescence kit (GE Healthcare Life Science, Germany).

Animals

Female DBA/2 mice were purchased at an age of 6–8 week from Harlan, Germany and kept under specific pathogen-free conditions in the animal facility of the Institut für Medizinische Strahlenkunde und Zellforschung, University of Wuerzburg, according to the guidelines. At the start of the immunization schedule, mice were approximately 10–14 weeks old.

Immunization procedures

Mice were immunized three times at an interval of 3 weeks. For the immunization with bacteria, animals were pretreated by applying 50 µl 7% $NaHCO_3$ intragastrically (p.o.) to increase the intragastric pH. About 5–10 min after pretreatment, 5×10^8 live, Kan-insensitive bacteria were applied in a volume of 100 µl PBS p.o. As a control, mice were immunized intramuscularly with naked plasmid DNA encoding for PSA (pcDNA3-PSA) as described elsewhere.[30]

ELISPOT

Seven days after the last immunization, splenocytes of immunized mice were prepared as published previously.[31] Enzyme-linked immunosorbent spot (ELISPOT) analysis for detecting PSA-specific $CD8^+$ T cells was performed according to a protocol published previously.[31] Briefly, for the *ex vivo* analysis of PSA-specific $CD8^+$ T cells, 96-well nitrocellulose plates (Millititer HA; Millipore, Bedford, MA) were coated with 5 µg ml^{-1} of the anti-mouse interferon (IFN)-γ monoclonal antibody (mAb) R4 (Pharmingen San Diego, CA) in 100 µl of carbonate buffer (pH 9.6). After overnight incubation at 4 °C and blocking with 1% BSA in PBS, 1×10^5 splenocytes from vaccinated mice were added in 100 µl RPMI 10 per well. For the analysis of the $CD8^+$ T-cell response the PSA-expressing P815 cell clone PPSA 18 was used. After incubation for 20–22 h at 37 °C, 5% CO_2 in the presence of 30 U ml^{-1} interleukin (IL)-2, plates were washed and incubated for additional 2 h with 100 µl biotinylated anti-mouse IFN-γ mAb XMB1.2 (0.25 µg ml^{-1}, Pharmingen). The plates were washed and incubated for 1 h at 37 °C in the presence of 100 µl of a 1/20 000 dilution of alkaline phosphatase-coupled streptavidin (Pharmingen). Spots were visualized by adding 50 µl of the ready-to-use substrate BCIP/NBT (Sigma, St Louis, MO) dissolved in water. Spots were counted under a dissecting microscope at threefold magnification. The frequency of peptide-specific T cells is represented by the number of IFN-γ-secreting cells per 10^6 splenocytes. For analysis of T-cell responses after restimulation, 2.5×10^7 splenocytes were restimulated with 2×10^6 irradiated PPSA 18 cells in RPMI 10 medium in the presence of 60 U ml^{-1} recombinant IL-2 for 5 days. ELISPOT analysis was performed as above, using various amounts of restimulated cells (10^5, 3×10^4, 10^4 or 3×10^3 per well) mixed with 4×10^5 feeder cells (freshly prepared splenocytes from naive DBA/2 mouse) and 10^5 PPSA 18 cells.

Protection against tumor cell challenge

To analyze the protective capacity of the immunization, 6–7 mice per group were immunized according to the schedule above. Two weeks after the third immunization, mice were challenged with PPSA 18 by two s.c. injection of 1×10^6 cells into each flank of shaven abdominal skin. Mice were monitored over a period of 14 days for tumor appearance and tumor volume was assessed by measuring the largest (a) and smallest (b) tumor diameter. Tumor volume was calculated as rotation ellipsoid using the following formula:

$$V = \frac{\pi}{6} * a * b^2, a > b.$$

The results' significances were analyzed by one-way analysis of variance (ANOVA), Dunnett's multiple comparison post test using the GraphPad Prism software. The post test was only performed when ANOVA revealed significance. Results are displayed as means ± s.d.

Results

Construction of recombinant S. typhimurium aroA *SL7207 strains carrying different PSA vaccines*

Ampicillin-resistant bacterial vaccines are inadequate for human use. Therefore, we started an approach to substitute the ampicillin resistance cassette of our base vaccine vector pMOhly1[20] by a kanamycin resistance cassette. The plasmid pMKhly1 was constructed as described in 'Materials and methods'. To test a possible adjuvant effect of CtxB we used pMKhly1 to generate different combinations between PSA and CtxB. In all constructs, the cancer antigen PSA was expressed and secreted as a hemolysin fusion protein (Figure 1). The plasmid pMKhly-PSA encodes a PSA–HlyAs fusion (Figure 1a). Plasmids pMKhly-CtxB and pMKhly-CtxB-PSA contain the CtxB fused to HlyAs or to PSA-HlyAs, respectively (Figure 1a). Finally, the plasmid pMKhly-PSA/CtxB contains, in addition to the PSA–HlyAs fusion, the *ctxB* gene under control of a constitutively

active tac promoter (Figure 1a). In all cases, with the exception of the pMKhly-PSA/Ctxb plasmid, both the *ctxB* gene and/or the *psa* gene were cloned without their original leader sequences. This is necessary since proteins containing both, an N-terminal signal sequence (for example, secretion type II or III) and the C-terminal HlyAs signal sequence are not efficiently transported across the membrane unless one of the competing protein transport machineries is inactivated.[32,33]

In the next step, the expression and secretion of the PSA fusion proteins in the *S. typhimurium* strain SL7207 were analysed by western blot (Figure 1b). The data showed that under these experimental conditions the amount of secreted PSA in all PSA-positive strains is similar and independent of the CtxB expression. Of note, we did not detect CtxB in the supernatant of SL7207/pMKhly-PSA/CtxB (Figure 1b(c) lane 6). The most probable reason might be that CtxB in *S. typhimurium* SL7207 is secreted into the periplasmic space via its own secretion signal where it forms pentamers and thus remains in the cell pellet (Figure 2b, lane 3). In contrast, secretion of the CtxB–PSA fusion protein via Type I secretion systems (T1SS) is very efficient (Figure 2a, lane 2) and assembly of the oligomeric structures of pentamer size was detected in the supernatant only (Figure 2a, lane 4).

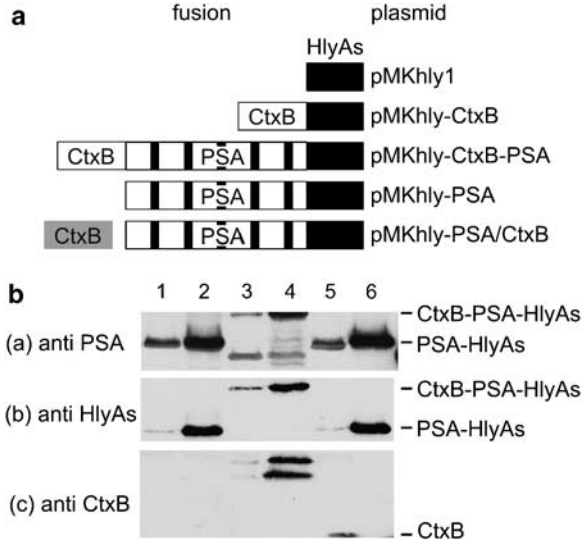

Figure 1 (**a**) Schematic representation of the fusion proteins encoded by the plasmids: pKMhly1, pMKhly-CtxB, pMKhly-CtxB-PSA, pMKhly-PSA and pMKhly-PSA/CtxB. The black boxes indicate the hemolysin signal (HlyAs), the white boxes the whole cholera toxin subunit B (CtxB) protein without the original leader sequence, the gray box the whole CtxB and the striated boxes the mature prostate-specific antigen (PSA). (**b**) Identification of the PSA fusion proteins by western blot. Cultures of *S. enterica* serovar Typhimurium SL7207 carrying the plasmids pMKhly-PSA (lanes 1 and 2), pMKhly-CtxB-PSA (lanes 3 and 4) and pMKhly-PSA/CtxB (lanes 5 and 6) were analyzed. Cellular proteins of 0.05 ml bacterial culture were loaded in lanes 1, 3 and 5; supernatant proteins precipitated from 2.5 ml of the bacterial culture were loaded in lanes 2, 4 and 6. The immunoblots were developed with polyclonal anti-PSA (**a**), anti-HlyAs (**b**) and anti-CtxB (**c**) antibodies.

PSA-specific CD8 response of DBA mice after immunization with recombinant SL7207 strains carrying PSA vaccines

The induction of a cellular immune response, especially $CD8^+$ T cells, plays an important role for the efficiency of tumor therapy.[34,35] Therefore, we first tested the efficacy of the recombinant bacterial strain SL7207 carrying PSA vaccines to induce a PSA-specific CD8 immune response. For this purpose, 64 female DBA/2 mice at the age of 10–14 weeks were immunized p.o. with recombinant SL7207/pMKhly-CtxB ($n=10$), SL7207/pMKhly-CtxB-PSA ($n=10$), SL7207/pMKhly-PSA ($n=10$), SL7207/pMKhly-PSA/CxtB ($n=10$), SL7207/pMKhly1 ($n=7$) and naked pcDNA3-PSA ($n=10$) as positive control as described in 'Materials and methods'. The ELISPOT data revealed that DNA-immunized mice showed profound T-cell responses (Figure 3). After restimulation, animals vaccinated with *Salmonella*-secreting PSA protein fused to CtxB displayed marked responses, whereas there was no significant response detected in those animals vaccinated with strains secreting PSA alone or PSA and CtxB separately (Figure 3). Interestingly, animals vaccinated with *Salmonella* secreting the CtxB toxin alone too showed significant responses after restimulation. Most likely this response is due to some cells of the innate immune system, which expand in the restimulation step and secrete IFN-γ. However, the nature of these cells is currently unknown.

PSA vaccines delivered by the S. typhimurium *SL7207 strain induced protection against tumors in a mouse model*

Two weeks after the final immunization, animals were inoculated with 10^6 PPSA 18 cells by s.c. injection in both sides of shaven abdominal skin, and tumor growth was monitored (Figure 4). Significant protective effects were

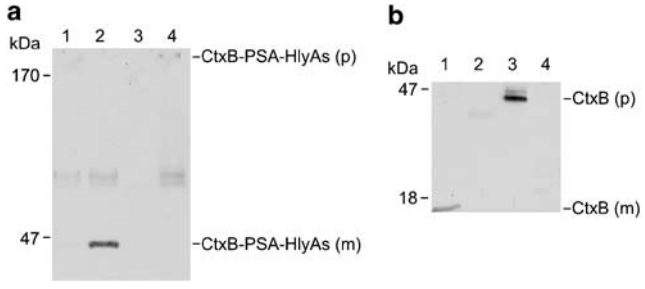

Figure 2 Identification of the mono- (m) and pentamer (p) of CtxB–Psa–HlyAs fusion (**a**) or cholera toxin subunit B (CtxB, **b**) by western blot. Cellular proteins of 0.05 ml cultures of *S. enterica* serovar Typhimurium SL7207 carrying the plasmids pMKhly-CtxB-PSA (**a**) or pMKhly-PSA/CtxB (**b**) were loaded in lanes 1 and 3. The supernatant proteins precipitated from 2.5 ml of same bacterial culture were loaded in lanes 2 and 4. For the analysis of pentamer formation, the proteins were taken up in 5× Laemmli buffer without β-mercapto-ethanol and were loaded without heating treatment (lanes 3 and 4). The immunoblots were developed with polyclonal anti-HlyAs (**a**) and anti-CtxB (**b**) antibodies.

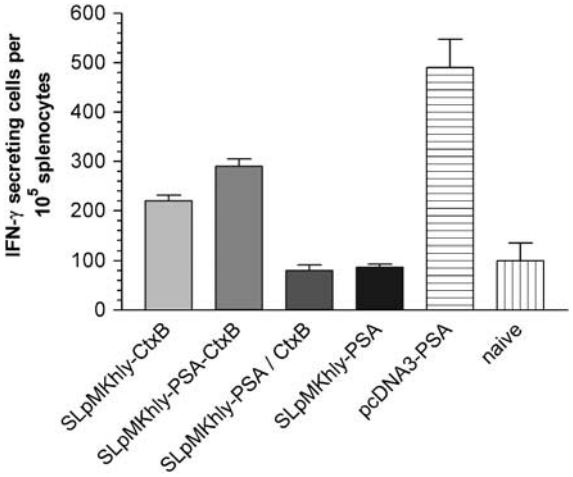

Figure 3 ELISPOT analysis of PPSA 18-specific cellular responses. Splenocytes from three mice per group were obtained 7 days after the last immunization as described, pooled and restimulated with PPSA 18. After 5 days of restimulation, cells were subjected to ELISPOT analysis. Mice immunized with *Salmonella* secreting cholera toxin subunit B (CtxB)–prostate-specific antigen (PSA), CtxB alone and mice immunized with naked DNA showed a significant increase in PPSA-reactive interferon (IFN)-γ-producing cells ($P<0.0001$; one-way ANOVA). In the *Salmonella* groups, strains secreting the CtxB–PSA fusion induced the most profound response ($P<0.001$), but also strains secreting CtxB alone induced significant numbers of IFN-γ-producing cells ($P<0.05$). In contrast, neither *Salmonella* alone nor *Salmonella* secreting PSA or PSA in addition to native CtxB induced detectable responses ($P>0.05$). Assays were performed in triplicate, mean ± s.d. is shown. Data were analyzed with one-way ANOVA and Dunnett's post test.

observed at days 6, 9, 12 and 14. As expected, naked DNA vaccination included as control completely protected mice from tumor growth. However, naked DNA vaccination shows moderate efficiency in humans. Regarding the bacterial constructs, the vaccine strain SL7207/pMKhly-CtxB-PSA turned out to be most efficient. It significantly reduced tumor volumes at days 9, 12 and 14 after tumor challenge. The vaccination with the SL7207/pMKhly-CtxB strain reduced tumor growth at day 14 with values comparable to that of SL7207/pMKhly-CtxB-PSA. Although not significant for SL7207/pMKhly-CtxB, this delayed effect is well compatible to the cellular response that was measured in the ELISPOT assay for this strain. Furthermore, also the SL7207/pMKhly-PSA strain achieved significant protection at day 14, which is in line with our previous observation in a lung cancer model and indicates that also this strain induced a T-cell response which was below the detection threshold. In contrast, the SL7207/pMKhly-PSA/CtxB strain did not induce any relevant effect and remained in the same range as SL7207/pMKhly1 alone.

Taken together, the SL7207/pMKhly-CtxB-PSA strain was the most efficient *Salmonella*-based vaccine in this experiment and showed significant protective effects starting from day 8 after tumor inoculation.

Discussion

In principal, two factors are crucial to the efficiency of a tumor vaccine: induction of a cellular immune response, especially $CD8^+$ T cells, and participation of components of the innate immune system, such as natural killer (NK) cells, NKT cells and γ-δ T cells.[34–36] In this context, it is widely known that bacterial carriers, especially *Salmonella* spp. can induce potent, protective humoral and cellular immune responses against the heterologous antigens[37,38] and can activate the components of the innate immune system.[39–41] This knowledge explains why bacteria like *Salmonella* represent promising vectors for delivery of tumor antigens. Until now, several *Salmonella* cancer vaccines, mainly DNA based, were developed, which are protective in different mouse cancer models.[38,42,43] However, DNA vaccines are inefficient in humans and there are no licensed bacterial vaccines for cancer therapy.

Here, we describe a new strategy for achieving an antitumor immune response with a PSA vaccine based on the recombinant attenuated *S. typhimurium aroA* SL7207 strain secreting a fusion protein of PSA and CtxB. In this study the PSA antigen in different combinations with CxtB was delivered in secreted form by the attenuated *S. typhimurium aroA* strain, using the *E. coli* hemolysin secretion system. This system allows an efficient antigen secretion and presentation, which leads to a more efficient antigen-specific CD8 immune response compared to the same antigen in a nonsecreted form.[21] In this context, very recently the type III secretion system (T3SS) was adapted for the delivery of cancer antigens into the cytosol of *Salmonella*-infected cells. This approach has proved efficient for priming antigen-specific $CD8^+$ T cells against tumor antigens.[44,45] All of these data demonstrated that if the heterologous antigen was delivered via the *Salmonella* carrier in a secreted form, it was able to induce CD8 response.

Concerning the efficacy of our prostate cancer vaccine in the mouse model, animals have been inoculated with the PSA-expressing tumor cell line PPSA and tumor growth was monitored. As expected, naive mice or mice immunized with the carrier strain alone showed progressive tumor growth over the whole observation period. Indeed, the observation period of 14 days was dictated by the tumor burden of the control animals.

In contrast, mice immunized with the *Salmonella* strain expressing the CtxB–PSA fusion efficiently decelerated tumor growth. Immunization with this strain significantly reduced tumor volume at days 9, 12 and 14 after tumor challenge. Of note, also the strain secreting CtxB alone reduced tumor growth with values comparable to the strain secreting the CtxB–PSA fusion at day 14. Although not significant, this delayed effect is well compatible to the innate cellular response which was measured in the ELISPOT assay and has already been described for CtxB or *Salmonella* alone.[15,46] Furthermore, also the PSA-secreting strain achieved significant protection at day 14, which indicates that also this strain induced a T-cell response which was below the detection threshold. In contrast, the strain separately expressing CtxB and PSA

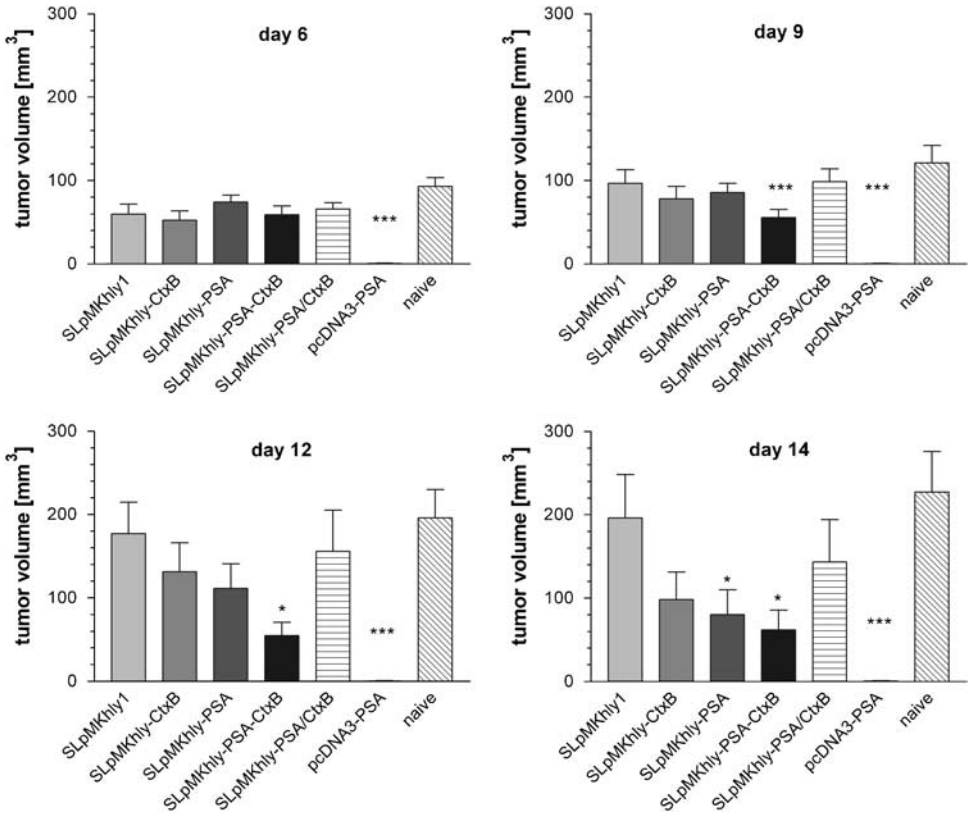

Figure 4 Tumor growth in immunized animals. Two weeks after final immunization, mice were inoculated with 10^6 PPSA 18 cells in each flank of shaven abdominal skin. Starting from day 3, tumor volume was assessed and first tumors appeared 6 days after tumor inoculation. Mice immunized with *Salmonella* secreting cholera toxin subunit B (CtxB)–prostate-specific antigen (PSA) fusion protein exhibited a significantly reduced tumor growth on days 9, 12 and 14. In contrast, mice immunized with *Salmonella* secreting PSA alone or CtxB showed a delayed response curve and mice immunized with PSA secreting strains showed a significant reduction of tumor growth at late time points, too. For analysis, both tumors of each mouse were included. Data were analyzed with one-way ANOVA and Dunnet's post test (*$P<0.05$, **$P<0.01$ and ***$P<0.001$).

did not induce any relevant effect distinguishable from the vector control.

The relative ineffectiveness of the strain expressing CtxB and PSA separately might be due to several reasons. First of all, CtxB is not expressed as a fusion protein to HlyA but rather as a native protein. Our western blot data demonstrated that the release of CtxB into the supernatant via its internal *sec*-dependent signal is, at minimum, very inefficient in *Salmonella*. Furthermore, although the tac-promoter has a comparable *in vitro* and *in vivo* activity,[47] the expression and the stability of CtxB *in vivo* might be lower than that *in vitro*. As a consequence, the extracellularly available level of native CtxB (in the form of pentamers) is very low and thus there is only negligible activity of CtxB as an immunostimulant. However, the secreted PSA of this strain should be able to induce a similar level of protection as the SL7207/pMKhly-PSA strain. A possible explanation is a different expression level of PSA in these strains *in vivo*.

The results observed with *Salmonella* secreting the CtxB–PSA–HlyAs fusion protein stand in sharp contrast: animals vaccinated with these bacteria show a significant decrease in tumor burden over the whole observation period. This result parallels the *in vitro* observations, where this strain turned out to be the most efficient inducer of PSA-specific cytotoxic T cells. Since this strain showed an efficient secretion of the fusion protein, as well as pentamer formation in western blot analysis, it is most likely that this strain forms the desired immunostimulatory complex leading to an efficient induction of antitumoral immunity. Taken together, these results suggest that the combination of two mucosal vaccines (adjuvants), namely *Salmonella* and CtxB, leads to a synergistic effect, which translates into the efficient induction of immunity with or without the additional expression of a specific antigen. Therefore, the use of *Salmonella* could successfully override the tendency of orally applied CtxB to induce systemic tolerance, and CtxB could markedly enhance the efficiency of *Salmonella* to induce cytotoxic T cells and innate immunity. The profound adjuvant effect observed with CtxB might be due to several reasons. In the case of CtxB, one can speculate that pentamerization, which in turn confers GM1-binding affinity, is the essential process that allows CtxB to act as a carrier for the fused antigen to cross the mucosal barrier and translocate into the cytoplasm of antigen-presenting cells, making the antigen more accessible to major histocompatibility complex (MHC) class I

presentation. The receptor-mediated mucosal uptake of CtxB seems to be an essential step required for its immunogenicity and strongly suggests that the adjuvant and carrier functions of CtxB are critically controlled by its binding affinity for cell receptors.[48] In this study, we demonstrated that our CtxB–Psa fusion protein can form heteropentameric fusion proteins under *in vitro* conditions. However, the question if the CtxB fusion proteins expressed and secreted in *S. typhimurium* can form pentamers *in vivo* is still open. On the other hand, MHC class II-restricted epitopes present on the CtxB subunit might stimulate CD4-restricted T helper cells that, in turn, increase the magnitude of the CD8 T-cell response. However, the exact mechanism of the CtxB-mediated adjuvant effect in our system is currently unknown.

This is the first example demonstrating that the T1SS is a versatile tool for the delivery of a combination of cancer antigens with CtxB in *S. enterica* serovar Typhimurium. Moreover, we have recently shown that this secretion system is also fully active in *S. enterica* serovar *typhi* Ty21a,[49,50] the only *Salmonella* vaccine strain registered for human use.[51] In addition *S. typhi* Ty21a can efficiently secrete this CtxB-PSA-HlyAs protein (Gentschev *et al*., in preparation). Hence, the combination of T1SS with CxtB and *S. typhi* Ty21a could represent the basis of a new generation of live bacterial vaccines against cancer in humans.

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Competing interests

The authors declare that they have competing interests.

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