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ORIGINAL ARTICLE

Development of a *Listeria monocytogenes* based vaccine against prostate cancer

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Abstract Prostate specific antigen (PSA) is a likely immunotherapeutic target antigen for prostate cancer, the second leading cause of cancer-related death in American men. Previously, we demonstrated that attenuated strains of *Listeria monocytogenes* (Lm) can be used as effective vaccine vectors for delivery of tumor antigens causing regression of established tumors accompanied by strong immune responses toward these antigens in murine models of cancer. In the present study, we have developed and characterized a recombinant live attenuated *L. monocytogenes*/PSA (*Lm*–LLO–PSA) vaccine with potential use for the treatment of pCa. Human PSA gene was cloned into and expressed by an attenuated *Lm* strain. This recombinant bacterial vaccine, *Lm*–LLO–PSA was tested for stability, virulence, immunogenicity and anti-tumor effects in a murine model for pCa. Immunization with *Lm*–LLO–PSA was shown to lower the number of tumor infiltrating T regulatory cells and cause complete regression of over 80% of tumors formed by an implanted genetically modified mouse prostate adenocarcinoma cell line, which expressed human PSA. *Lm*–LLO–PSA was immunogenic in C57BL/6 mice and splenocytes from mice immunized with *Lm*–LLO–PSA showed significantly higher number of IFN-γ secreting cells over that of the naïve animals in response to a PSA H2Db-specific peptide, as measured by both, ELISpot and intracellular cytokine staining. In addition, using a CTL assay we show that the T cells specific for PSA were able to recognize and lyse PSA-peptide pulsed target cells in vitro. In a comparison study with two other PSA-based vaccines (a pDNA and a vaccinia vaccine), *Lm*–LLO–PSA was shown to be more efficacious in regressing established tumors when used in a homologues prime/boost regimen. Together, these results indicate that *Lm*–LLO–PSA is a potential candidate for pCa immunotherapy and should be further developed.

Keywords *Listeria monocytogenes* · PSA · Cancer vaccine · Immunotherapeutic · Listeriolysin O

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Introduction

Prostate cancer (PCa) is the second most common type of cancer among men in USA. Only skin cancer is more common, and only lung cancer is responsible for more cancer-related deaths (Cancer facts and figures, American cancer Society, http://www.cancer.org). One out of every three men who are diagnosed with cancer each year has PCa [1]. Early PCa usually has no symptoms and is most commonly detected through PCa screening tests. The standard therapies for PCa include surgery, brachytherapy, radiation and hormone therapies, each associated with several adverse effects with an overall 10–30% chance of recurrence [2]. Recently, the development of cancer vaccines has offered the promise of a safer and more effective alternative to conventional cancer therapy. Immunotherapy mediated through cytotoxic T lymphocytes (CTL) provides a potential approach to cancer treatment, because T cells, in principle, can migrate throughout the body and specifically

recognize and destroy metastatic tumor cells in an antigen-specific manner [3]. At present, a preferred strategy for fighting PCa is to activate cytotoxic T lymphocytes against a specific antigen, which is ideally overexpressed by the tumor cells and not presented or expressed at very low levels by other tissues.

A number of prostate associated antigens have been reported in the literature [4]. One of the best candidates is prostate-specific antigen (PSA), which is a kalikrein serine protease (KLK3) secreted by prostatic epithelial cells [5] and is widely used as a serum marker for PCa [6]. PSA has potential as a target for immunotherapy against pCa, because it is produced at low levels by normal epithelial cells of the prostate gland, but is over-expressed in malignant tissue and not expressed by other tissues except for very low levels in small intestine and testis [7]. Anti-PSA cytotoxic and antibody responses have been detected in healthy men and patients with prostate tumors. There are a large number of studies discussing its potential as a target antigen against PCa [8–12].

Listeria monocytogenes (*Lm*) is an intracellular bacterial pathogen, which has been used as a delivery vehicle for various antigens [13–15]. *L. monocytogenes* has access to both phagosomal and cytosolic compartments and thus antigens delivered by *Lm* can be presented in the context of both MHC I and II molecules, resulting in strong cellular-immune responses [16]. Our previous studies have indicated that when fused to a truncated form of Listeriolysin O (LLO), antigens expressed and secreted by *Lm* are significantly more immunogenic than when they are secreted as stand alone proteins [13, 15]. Based on this, we constructed a new vaccine strain (*Lm*–LLO–PSA) comprised of an attenuated *Lm*, which expresses and secretes a fusion of LLO to human PSA. This vaccine was shown to cause regression of established PSA expressing solid tumors in mice. This effect was accompanied by strong and specific cellular immune responses toward PSA.

Materials and methods

Oligos and pCDNA3.1 were purchased from Invitrogen (Carlsbad, CA, USA) and DNA sequencing was performed by Genewiz Inc (Plainfield, NJ, USA). Peptides were synthesized by EZbiolabs (Westfield, IN, USA). Flow cytometry reagents were purchased from Becton Dickinson (BD) Biosciences (San Diego, CA, USA). Cell culture media and supplements were from Gibco/Invitrogen. ELISpot antibodies were purchased from Mabtech AB (Cincinnati, OH, USA). Other reagents, unless indicated, were from Sigma (St. Louise, MO, USA). PSA/vaccinia was kindly provided by Dr. Schlom at National Cancer institute, Bethesda, MD, USA.

Mice, cell lines and media

C57BL/6 mice were purchased from Jackson Laboratories (Bar Harbor, ME). Mice were maintained at Cook Campus animal facility at Rutgers University, New Brunswick, NJ, USA. Experiments on mice were performed in accordance with regulations by the Institutional Animal Care and Use Committee of Rutgers University. All cell lines were purchased from ATCC (Manassas, VA, USA). TRAMPC-1 (*T*ransgenic *A*denocarcinoma of the *M*ouse *P*rostate) cell line, derived from a C57BL/6 mouse, has been previously described [17]. Cells were grown and maintained in Dulbecco's modified Eagle's medium with 4 mM L-glutamine adjusted to contain 1.5 g/L sodium bicarbonate and 4.5 g/L glucose supplemented with 5 μg/ml bovine insulin, 10 nM dehydroisoandrosterone, 5% fetal bovine serum and 5%Nu-Serum IV. MC57G fibrosarcoma cells (from C57BL/6 background) were maintained in Eagle's minimum essential medium (EMEM) with 2 mM L-glutamine and Earle's BSS adjusted to contain 1.5 g/L sodium bicarbonate, 0.1 mM non-essential amino acids, and 1.0 mM sodium pyruvate and 10% fetal bovine serum. EL4 lymphoma cells were maintained in Dulbecco's modified Eagle's medium with 4 mM L-glutamine adjusted to contain 1.5 g/L sodium bicarbonate and 4.5 g/L glucose, 10 % fetal bovine serum. J774A.1, a murine macrophage cell line was maintained in RPMI1640 medium with 4 mM L-glutamine adjusted to contain 1.5 g/L sodium bicarbonate and 4.5 g/L glucose and 10% fetal bovine serum. Complete RPMI (C-RPMI) medium contained RPMI 1640 complemented with 2 mM glutamine, 0.1 mM non-essential amino acids, and 1.0 mM sodium pyruvate and 10% fetal bovine serum.

Construction of PSA/pCDNA3.1 vaccine

The entire open reading frame of PSA including its signal sequence was cloned into pCDNA3.1$^{(-)}$ vector backbone (Invitrogen) at *Xba*I and *Xho*I sites. In this plasmid, expression of PSA was under the control of CMV promoter and was confirmed by transient transfection into 293-cell line (ATCC). Secretion of PSA by transfected cells was confirmed in the supernatant of cultured cells using an anti-PSA ELISA kit (American Qualex, San Clemente, CA, USA). Plasmid GM-CSF/pCDNA was a kind gift from Dr. Vonderheide, University of Pennsylvania, Philadelphia, PA, USA.

Construction of *Lm*–LLO–PSA vaccine

The gene encoding for human PSA (excluding its secretion signal sequence) was amplified using *pfx* polymerase, forward oligo: gtgCTCGAGattgtgggaggctgggagtg, and the reverse oligo: gatACTAGTttaggggttggccacgatgg. The

two restriction sites, *Xho*I and *Spe*I that were used for in frame cloning of PSA as a fusion to LLO in pGG55 [13] are indicated in capital letters. The stop codon to terminate the translation of LLO–PSA fusion protein is underlined in the reverse oligo. Amplified PSA was cloned into the *Lm* plasmid pGG55, as a fusion protein to the first 441 amino acids at the N-terminal of LLO (pAdv34, Fig. 1), between the restriction sites XhoI and SpeI [18]. The resulting plasmid was sequenced to assure the absence of mutations. The plasmid pAdv34 was electroporated into *Lm* strain XFL7 (which lacks the genomic *prfA* gene) and was originally derived from the streptomycin resistant strain *Lm* 10403 s, as described earlier [13]. *Listeriae* containing the plasmid (*Lm*–LLO–PSA) were selected using chloramphenicol (34 μg/ml) and 250 μg/ml of Streptomycin on Brain Heart Infusion (BHI/Chl/S) plates. Colonies were screened by PCR to confirm the presence of the PSA gene.

Expression of LLO–PSA by *Lm*–LLO–PSA

Lm–LLO–PSA colonies were grown in BHI/Chl/S broth at 37°C and 225 rpm for 8 h. Bacteria were separated from the culture medium by centrifugation at 10,000*g* for 5 min and the supernatants recovered. Proteins in the culture supernatant were precipitated for at least 1 h in the presence of 10% v/v of tri-chloroacetic acid at 4°C and separated on a 4–20% gel by SDS-PAGE. After transferring the proteins onto PVDF membranes, LLO–PSA fusion protein was detected by immunoblotting with either rabbit polyclonal anti-LLO (made in house) or anti-PSA antibodies (Sigma). Signals were developed using the Western-Breeze chromogenic Kit (Invitrogen).

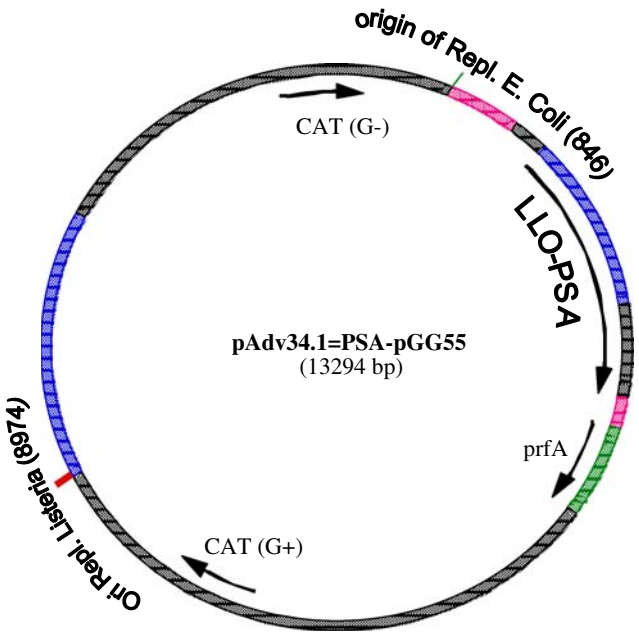

Fig. 1 Schematic map of pAdv34 in pGG55. CAT(G−), chloramphenicol acetyltransferase for *E. coli*; CAT(G+), chloramphenicol acetyltransferase for *Lm*; Ori, replication region; *prfA*, *Lm* pathogenicity-regulating factor A; LLO-PSA, fusion between the gene encoding a C-terminally truncated listeriolysin O, including its promoter, and the gene encoding human PSA

In vivo passaging of *Lm*–LLO–PSA

Lm–LLO–PSA was passaged in vivo two times as described by Peters et al. [19]. Briefly, a dose of 1×10^6 CFUs of *Lm*–LLO–PSA was injected intraperitoneally (i.p.) to 6–8 week old mice. Spleens were harvested 3 days after the injection; single cell suspensions were prepared from the spleens and plated on BHI/Chl/S plates to select for *Lm*–LLO–PSA growth. This procedure was repeated one more time using a LLO-PSA positive colony from the first passage. *Lm*–LLO–PSA colonies recovered after two in vivo passages were tested for LLO-PSA secretion. A single clone was chosen to be used for plasmid DNA sequencing, tumor studies and other functional assays.

In vivo virulence study

A virulence study was performed to determine a safe dose of *Lm*–LLO–PSA in mice. Two groups of 4 male, C57BL/6 mice (7 weeks old) were injected i.p. with two different doses of *Lm*–LLO–PSA: 1×10^8 and 5×10^7 CFUs/dose. Mice were monitored for signs of sickness for up to 5 days post inoculation.

Plasmid sequencing

Lm–LLO–PSA was grown overnight in 200 ml of BHI/Chl/S at 37°C and 225 rpm. Bacteria were separated from culture broth by centrifugation at 10,000*g* at 4°C for 10 min. Bacterial pellet was treated with lysozyme (2 mg/ml) at 37°C for 30 min to break the cell wall. The plasmid pAdv34 was purified using PureLink plasmid midiprep kit (Invitrogen) according to manufacturer's instructions and entirely sequenced by the dideoxi chain terminator method.

In vitro macrophage invasion study

Murine macrophage-like J774A.1 cells were plated at 1×10^6 cells per well of 24-well plates. The day after, cells were infected at MOI of 1:1 with different *Lm* strains for 1 h in the absence of antibiotics. Extracellular bacteria were eliminated by washing and incubating the cells in medium containing 50 μg/ml of gentamicin for 30 min at 37°C and 5% CO_2. The intracellular bacterial growth was monitored

by taking samples at different time points for up to 8 h followed by titration of the bacteria on BHI plates after lysing the cells with dH_2O.

Anti-tumor efficacy

TRAMPC-1 is a murine adenocarcinoma prostate tumor cell line on the C57BL/6 mouse background [17] and was used to construct a PSA secreting prostate tumor model for testing the efficacy of *Lm*–LLO–PSA as a vaccine. The complete human PSA open reading frame, including its secretory signal sequence at the N-terminal of the molecule was cloned under the control of the UbC promoter in the plasmid pUB6/V5 (Invitrogen). The resulting plasmid (pAdv63) was sequenced for the confirmation of the correct PSA gene sequence and then transfected into the TRAMPC-1 cell line using lipofectamine 2000 (Invitrogen). Transfected cells were selected by culturing the cells in medium containing 10 μg/ml of blasticidin (Invitrogen). Single clones were isolated by the limiting dilution method and were screened for the secretion of PSA into the extracellular culture medium using an anti-human PSA ELISA kit. Several positive clones were obtained, expanded and stored in liquid nitrogen. One clone (T-PSA23) was selected for further studies.

Tumor regression study

Three groups of 8 male C57BL/6 mice (6–8 week old) were inoculated s.c. in the right flank with 5×10^6 T-PSA23 cells. Seven days later, when tumors reached an average diameter of 4–5 mm, they were immunized i.p. with *Lm*–LLO–PSA (1×10^7 CFUs/dose), *Lm*-LLO-E7 (1×10^8 CFUs/dose) or were not treated. *Lm*-LLO-E7 previously described by Gunn et al. [13], was constructed in the same manner as *Lm*–LLO–PSA, but instead of PSA, it expressed HPV16E7 and was used in this experiment as an irrelevant *Lm* control. Immunizations were repeated twice with 1-week intervals. Tumors were monitored weekly for 7 weeks. Blood samples from all experimental animals were collected on day 40 post-tumor inoculation from the tail vein and the presence of PSA protein in the sera was tested using the PSA ELISA kit. For comparison studies, tumor cells were injected as described above. Mice were immunized twice with *Lm*–LLO–PSA (1×10^7 CFUs/dose in 200 μl PBS), a recombinant PSA-vaccinia (1×10^7 PFUs/dose in 200 μl PBS, i.p.) or a pDNA vaccine comprising of a mixture of PSA/pCDNA3.1 (100 μg) with GM-CSF/pCDNA3.1 (100 μg) in 50 μl volume, by intramuscular injection in the left quadriceps. Tumors were monitored once a week using an electronic caliper and tumor sizes were expressed as the mean of two perpendicular diameters.

Immunogenicity studies in mice

ELISpot assay

Groups of 4–5 male C57BL/6 mice (6 to 8-weeks old) were immunized three times with either *Lm*–LLO–PSA or a similar construct containing an irrelevant antigen as negative control (*Lm*-LLO-WT1), or were not immunized (naïve). Six days after the boost, spleens were harvested and processed to a single cell suspension. Red blood cells were lysed by 2 min incubation with ACK lysing solution followed by two washes with C-RPMI medium. Isolated splenocytes were plated at 2×10^5 cells/well in the presence of 1 μM $PSA_{65–74}$ peptide ($H2D^b$–restricted PSA epitope HCIRNKSVIL) [20] on ELISpot plates previously coated with mouse IFN-γ antibody (mAb AN18). Plates were incubated overnight at 37°C. Spot forming cells (SFC) were detected according to manufacture's instructions (Mabtech AB). Spots were counted using a dissecting microscope.

Intracellular staining for IFN-γ

Splenocytes isolated from the immunized, control or naïve mice were stimulated at 2×10^6 cells/well for 5 h in round bottom 96-well plates with 1 μM of either $PSA_{65–74}$ peptide or control peptides in the presence of IL-2 (50 U/ml) and monensin A (StopGolgi, BD Biosciences). Cells incubated in the medium without peptide were used as negative control. After incubation, cells were stained with anti-mouse CD8-FITC, CD3-PerCP-Cy5.5 and CD62L-APC antibodies. Subsequently, cells were permeabilized and stained with anti-mouse IFNγ-PE antibody and analyzed in a FACS Calibur (BD Biosciences) cytometer. The data were analyzed using CellQuest Pro software. Cells were gated as $CD3^+CD8^+CD62L^{low}$ and the number of IFN-γ-positive cells expressed as a percentage of total gated cells.

Cytotoxicity assay

The cytotoxic activity of T cells in response to *Lm*–LLO–PSA vaccination was measured by a PSA specific CTL assay. Groups of 4 male C57BL/6 mice were injected i.p. with three doses of *Lm*–LLO–PSA (1×10^7 CFUs) or a *Listeria* expressing an irrelevant antigen (HPV16E7E6), with 1 week intervals. Naïve group did not receive any immunization. Spleens of the immunized and naïve mice were harvested 6 days after the last boost. Splenocytes were incubated for 5 days in C-RPMI medium containing 20 U/ml of mouse IL-2 (Sigma), in the presence of MC57G cells infected with PSA/vaccinia (MOI 5 for 4 h) and treated with mitomycin C at an effector: stimulator ratio of 20:1. The cytotoxicity assay was performed for 4 h by incubating the effector cells with EL4 target cells, pulsed for 1 h with the $H2D^b$, $PSA_{65–74}$ peptide and labeled with

europium [21]. Europium released from the lysed cells was measured by mixing a sample of the cell culture supernatant with europium enhancement solution (Delfia/Perkin Elmer, Waltham, MA, USA) and an absorbance reading at 590 nm (Perkin Elmer/Wallac, VictorII). Two sets of assays were performed: (a) using different effector: target (E:T) ratios at a fixed peptide concentration of 1 μM; and (b) using different concentrations of the PSA peptide at a fixed E:T ratio of 25:1. SC_{50} values were calculated as the peptide concentration required for 50% of the value of maximum peptide concentration used (1 μM) minus the background level at 0 μM.

Analysis of tumor infiltrating lymphocytes (TILs)

T-PSA23 cells embedded in matrigel were inoculated s.c. in the right flank of male C57BL/6 mice, which were immunized on days 7 and 14 with *Lm*–LLO–PSA, a control *Lm* vaccine or left untreated. On day 20 the tumors were surgically excised, washed in ice-cold PBS and minced with a scalpel. The tumor was incubated in matrigel cell recovery solution (BD Biosciences) for 2 h on ice. TILs were further released by mechanical disruption of the tissue in a cell strainer using a syringe plunge. Isolated cells were stained with a $PSA_{65–74}$ H-$2D^b$ tetramer-PE and anti-mouse CD8-FITC, CD3-PerCP-Cy5.5 and CD62L-APC antibodies to characterize CTLs specific for the PSA epitope. To analyze regulatory T cells in the tumor, TILs were also stained with CD4-FITC, CD3-PerCP-Cy5.5 and CD25-APC. Cells were subsequently permeabilized and stained with anti-FoxP3-PE antibody (Milteny Biotec). Cells were analyzed in a FACS Calibur (BD Biosciences) cytometer. The data were analyzed using CellQuest Pro software. Regulatory T cells were defined as $CD3^+CD4^+CD25^+Foxp3^+$ cells.

Statistical analysis

Non-parametric Mann–Whitney and Kruskal Wallis tests were applied to compare tumor sizes among different treatment groups. Tumor sizes were compared on day 40 for statistical analysis, because this was the latest time-point with the highest number of mice in each group. The Student's *t* test was used to compare the means in the ELISpot experiments. A *P* value of less than 0.05 was considered statistically significant in these analyzes.

Results

Generation of recombinant *L. monocytogenes* expressing PSA

The gene encoding for human PSA was cloned as a fusion protein with a truncated, non-hemolytic segment of LLO, which contains a conserved PEST sequence at its N terminal [22–24] (Fig. 1). The plasmid backbone pGG55 as previously described [13], allows for the expression and secretion of LLO fusion proteins from an episomal origin by the attenuated *Lm* strain XFL7, which lacks the genomic virulence factor *prfA* [25]. The *prfA* gene is required for the in vivo survival of *Lm* and thus, is complemented by a copy carried on the plasmid. The PSA signal sequence was deleted to avoid any intervention of this sequence with the proper secretion of the LLO-PSA fusion protein, which is mediated by the LLO secretion signal sequence in *Lm*–LLO–PSA.

Lm–LLO–PSA can express and secrete LLO-PSA fusion protein

Expression and secretion of the LLO-PSA fusion protein from *Lm* was tested in the cell culture supernatants after in vitro growth of the bacteria. We tested four colonies of *Lm*–LLO–PSA and all were able to secrete the fusion protein LLO-PSA as detected with both anti-LLO and anti-PSA antibodies (Fig. 2). This secretion was maintained in bacteria isolated after two in vivo passages in mice (data not shown), suggesting that the plasmid is stable and retained in vivo for at least three days. In order to confirm stability, pAdv34 was extracted from the passaged *Lm*–LLO–PSA and was entirely sequenced. There were no mutations detected in the plasmid backbone or the PSA gene after two in vivo passages, as compared to our reference plasmid pGG55 [13] and the human PSA gene sequence (NCBI: NM_001648). To determine a safe dose of *Lm*–LLO–PSA to be used for the animal studies, two doses of this construct were tested in mice by i.p. injection (1×10^8 and 5×10^7 CFUs/dose). The dose used in the animal experiments (1×10^7 CFU/mouse) was defined as one-tenth of the lowest dose observed to have deleterious effect on the immunized mice.

Lm–LLO–PSA is able to infect macrophages in vitro

Uptake by antigen presenting cells such as dendritic cells and macrophages is an essential requirement for *Lm* based vaccines to successfully deliver and present antigens into the immune system [26]. This property can be tested in vitro by infecting the murine macrophage-like cell line J774A.1 and quantifying the intracellular bacterial growth, which is an indication that the *Lm* vaccine strain has been able to properly enter the cells and multiply. In this assay, *Lm*–LLO–PSA was shown to be able to invade macrophages and grow in a similar manner to the wild type *Lm* strain 10403 s. In contrast, the *Lm* strain XFL7, which lacks *prfA* and does not escape the phagolysosome, is not able to proliferate in J774A.1 cells and its titer remains constant

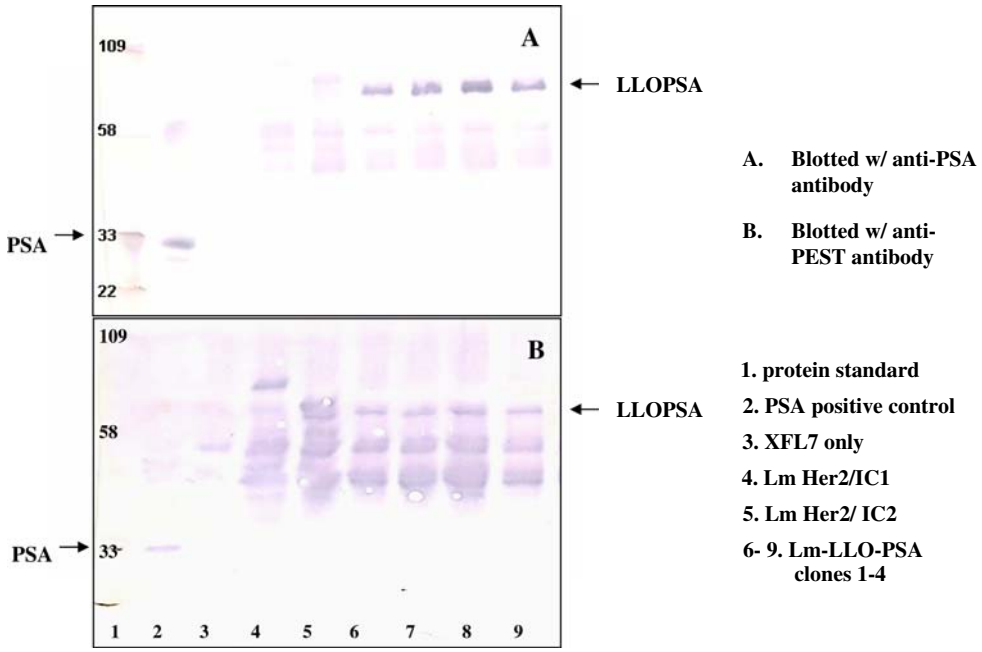

Fig. 2 Expression and secretion of LLO-PSA fusion protein by *Lm*–LLO–PSA. Bacteria were grown overnight and cell supernatants were precipitated in the presence of 10% TCA at 4°C. Proteins were separated by SDS-PAGE and blotted with anti-PSA (**a**) and anti-LLO (**b**) antibodies. PSA positive control was a BscI cell lysate infected with PSA/vaccinia. The parent bacterial strain XFL7 and two irrelevant *Lm* strains expressing fragments of human Her2/neu antigen were used as negative controls

several hours post-infection (Fig. 3). The complementation of *prfA* in *Lm* strain XFL7 with the plasmid pAdv34 resulted in the restoration of the in vitro invasive properties of the strain and this was comparable to those of the wild type strain.

Lm–LLO–PSA causes regression of pre-established PSA expressing tumors in mice

The efficacy of *Lm*–LLO–PSA in regressing PSA expressing tumors was tested in a mouse model using a PSA-transfected TRAMPC-1 cell line. TrampC-1 cells, originally derived from a murine prostate adenocarcinoma tumor [17], were stably transfected with the human PSA gene under the control of the human Ubiquitin C promoter. Secretion of PSA by transfected cells was confirmed by ELISA. Four clones were expanded and passaged for 25 generations in the absence of the antibiotic selection marker, blasticidin. Cell culture supernatants were tested for the levels of PSA secretion which were shown to be comparable between the cells at early passage and passage 25 (data not shown). One of these clones retained tumorgenicity in vivo and formed solid tumors upon s.c. inoculation in male C57BL/6 mice. An early passage of this clone was used for the tumor studies and it is referred as T-PSA23. Three groups of 8 mice were inoculated s.c. with 5×10^6 T-PSA23 cells and were monitored for tumor growth daily. After 7 days, a solid tumor mass of 4–5 mm in diameter was detected in 7 of 8 mice in each group (Fig. 4). At this time mice were immunized with *Lm*–LLO–PSA (1×10^7 CFUs/mouse), *Lm*-LLO-E7 (1×10^8 CFUs/mouse) or left untreated. Primary immunization was followed by two booster doses at 1-week intervals with the same dose as the prime. Mice (7 of 8) in each of the control groups developed slow growing tumors, which reached a diameter of 2 cm within 7 weeks post-inoculation (Fig. 4), at which point they were sacrificed. One mouse in each group did not show any signs of tumor at any time. Five days after the first immunization, tumor reduction was observed in 7 of 8 of *Lm*–LLO–PSA immunized group and complete tumor regression was observed in 5 of these mice after 1 week. One of the tumors in the *Lm*–LLO–PSA immunized group remained stable until the third immunization. However, 1 week after the third

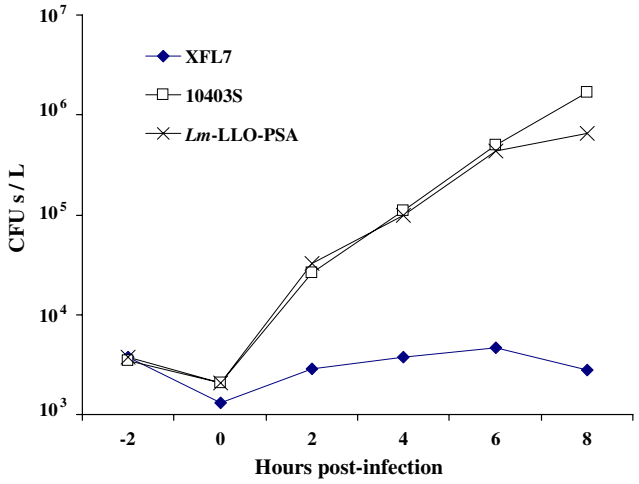

Fig. 3 Uptake and growth of *Lm*–LLO–PSA in macrophages. Murine macrophages like cells (J774A.1) were infected in vitro with MOI of 1:1 with *Lm*–LLO–PSA, the parent *Lm* XFL7 or the wild type *Lm* 10403 s strains. The intracellular growth was monitored for 8 h by taking duplicate samples every other hour from the cells followed by titration of the bacteria on BHI plates. CFUs: Colony Forming Units

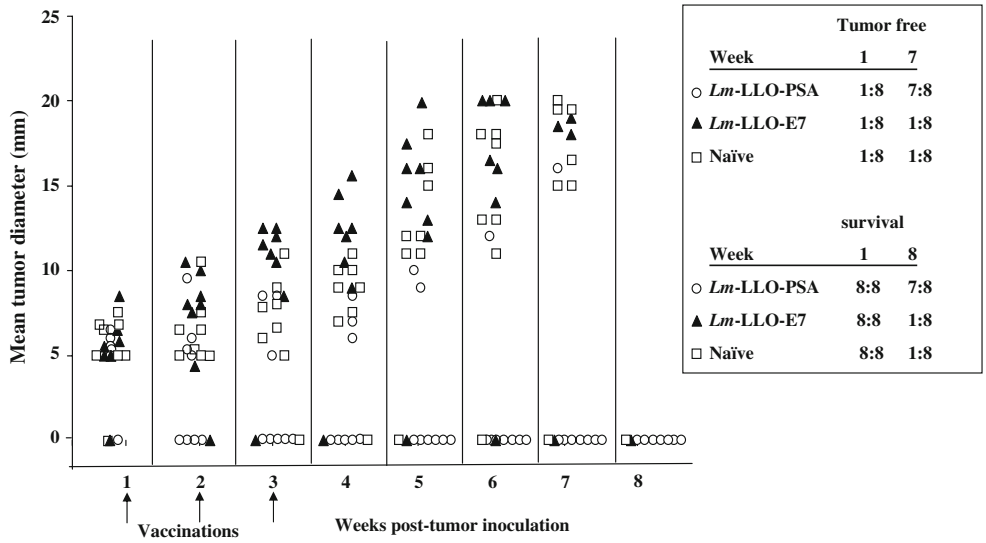

Fig. 4 Tumor regression upon *Lm*–LLO–PSA immunization. Groups of 8 mice were inoculated s.c. with 5×10^6 T-PSA23 cells on day 0 and immunized three times with 1 week intervals, i.p. with *Lm*–LLO–PSA, *Lm*-LLO-E7 or received no immunization (naïve). Tumors were monitored weekly using an electronic caliper and are expressed as the mean of two perpendicular diameters. Mice were sacrificed when the tumors reached a size of 15–18 mm in diameter. Results are depicted as tumor size from each individual mouse. The table shows the number of mice in each group, which were either tumor free (*upper panel*) or survived (*lower panel*) relative to the total number of mice per group 1 week or 7–8 weeks post-tumor inoculation. This experiment was repeated three time showing similar results

vaccination, this tumor also regressed and became undetectable. Another mouse in this group developed a slow growing tumor, which reached a size of 18–20 mm within 7 weeks and was sacrificed. At the end of the study on week 8 post-tumor inoculation, 7 of 8 mice in the *Lm*–LLO–PSA immunized group were still tumor free. This experiment was repeated three times with similar results. Non-parametric Kruskal–Wallis statistical analysis was performed on the tumor sizes on day 40, before mice in naïve and *Lm*-LLO-E7 groups were sacrificed. There were no statistically significant differences among the tumor sizes of the mice in the naïve group and the mice immunized with *Lm*-LLO-E7 ($P = 0.613$). However, immunization with *Lm*–LLO–PSA was shown to make a significant impact on the T-PSA23 tumor growth ($P = 0.002$). Serum samples from controls and *Lm*–LLO–PSA immunized mice were collected on day 40 post-tumor challenge and tested for the presence of PSA using an anti-human PSA ELISA kit (American Qualex). Significant levels of PSA (5–10 ng/ml) were detected in the serum of control mice and in the mouse in the *Lm*–LLO–PSA group, which had tumors. No PSA was found in the serum of *Lm*–LLO–PSA immunized mice after tumor regression.

Lm–LLO–PSA elicits cellular immune responses against PSA in both spleens and tumors

To elucidate whether vaccination with *Lm*–LLO–PSA induced PSA-specific CTLs able to infiltrate the tumor, mice were implanted s.c. with a mixture of T-PSA23 cells + matrigel and immunized with two doses of *Lm*–LLO–PSA, *Lm*-LLO-E7 or none. Spleens and tumors were harvested 6 days after the last immunization and tested for the presence of $CD8^+/PSA_{65–74}(H2D^b$ restricted) tetramer positive T cells by FACS analysis. No significant anti-PSA responses were observed in spleens of naïve (0.05%) or *Lm*-LLO-E7 (0.09%) vaccinated mice (Fig. 5, upper panel). However, upon immunization with *Lm*–LLO–PSA, a significant number of PSA specific $CD8^+$ T cells were found in the spleens. Interestingly, when we tested the tumors excised from these mice for the presence of TILs, low number of PSA specific $CD8^+$ T cells were identified in both naïve and *Lm*-LLO-E7 immunized mice (1.5 and 0.51%, respectively). Immunization with *Lm*–LLO–PSA caused a considerable increase in this number as 16.1% of all $CD8^+$ TILs were shown to be $PSA_{65–74}$ specific (Fig. 5 lower panel).

Immunization with *Lm*–LLO–PSA causes a decline in the T regulatory cells in the tumors but not in spleens

The presence of $CD4^+/CD25^+/FoxP3^+$ T regulatory (Treg) tumor infiltrating cells have been shown to be associated with increase chance of tumor progression. Therefore, we examined the presence of this T cell population in tumors of *Lm*–LLO–PSA immunized or control mice. Interestingly, immunization with either *Lm*–LLO–PSA or *Lm*-LLO-E7 caused a considerable decrease in the frequency of Tregs in the tumors as compared to tumors from naïve mice (Fig. 6). In fact, *Lm*–LLO–PSA had slightly more impact than *Lm*-LLO-E7 in the frequency of tumor infiltrating Tregs. On the

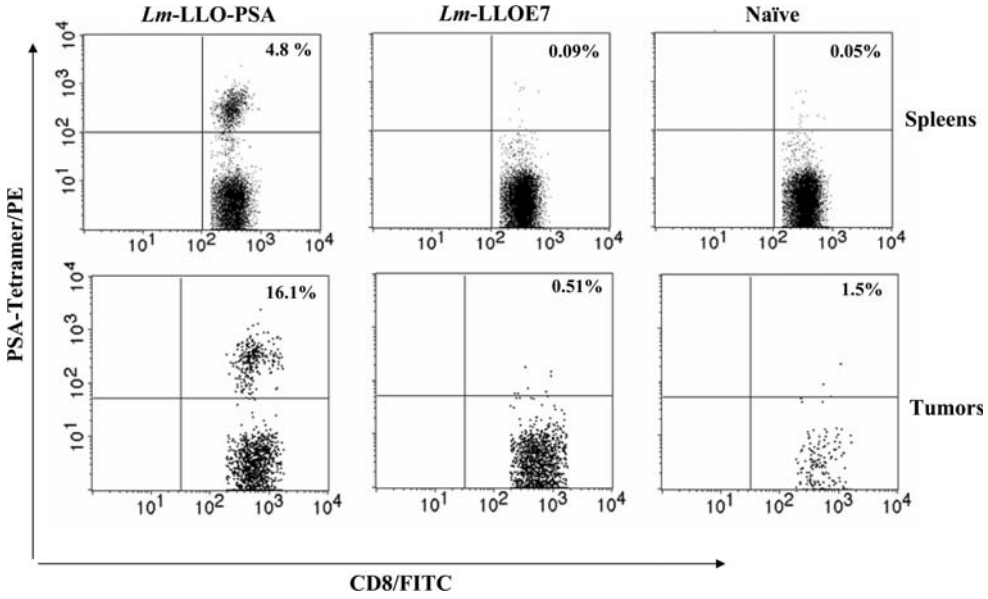

Fig. 5 PSA specific CD8+ T cells in spleens and tumors. T-PSA23 cells were implanted s.c. as a mixture with matrigel. Mice were immunized twice with *Lm*–LLO–PSA or *Lm*-LLO-E7 or were left naïve. Spleens and tumors were extracted 6 days after the second immunization and tested for the presence of $CD8^+$/PSA tetramer positive T cells by FACS analysis. *Lm*–LLO–PSA immunizations resulted in the generation of PSA specific $CD8^+$ T cells in both spleens (*upper panel*) and tumors (*lower panel*). Numbers in each corner indicated the % of PSA positive cells over total number of $CD8^+$ T cells in each tissue

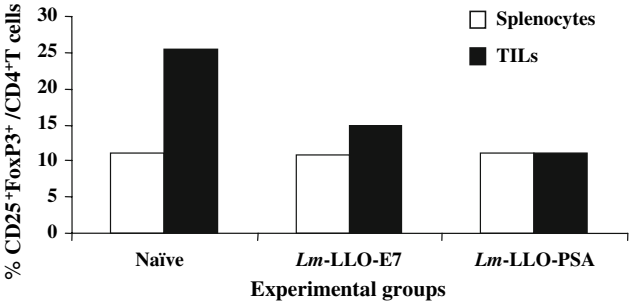

Fig. 6 Effect of vaccination with *Lm* vaccines on the presence of Tregs in spleens and tumors. Isolated splenocytes and TILs extracted from 3 tumor bearing mice in the previous experiment were pooled and stained for CD4, CD25 and FoxP3 to elucidate the effect of immunization with *Lm*–LLO–PSA on the presence of Tregs in these tissues. Each column presents the % of $CD25^+/FoxP3^+$ T cell population in relation to the total $CD4^+$ T cells in each pool

other hand, there were no significant differences between the percentages of $CD4^+/CD25^+/FoxP3^+$ T cells isolated from the spleens of *Lm*–LLO–PSA or control mice.

Immunization with *Lm*–LLO–PSA results in strong cellular immune responses against PSA

The immune responses elicited by *Lm* based vaccines have been shown to result in cellular, rather than humoral responses [16]. We investigated the activation and functionality of $CD8^+$ T cells induced by *Lm*–LLO–PSA in spleens of immunized mice.

Cytokine secretion

Secretion of IFN-γ by activated T cells in response to an in vitro stimulation with $H2D^b$ PSA peptide was tested by ELISpot and intracellular cytokine staining. Mice were immunized three times with *Lm*–LLO–PSA or *Lm*-LLO-WT-1 (*Lm*-LLO-Wilm's Tumor Antigen as negative control) or were not immunized (naïve). Isolated splenocytes were then prepared and incubated with the PSA peptide and the IFN-γ secreting $CD8^+$ cells were quantified. In both assays, mice immunized with *Lm*–LLO–PSA showed a significantly higher number of IFN-γ secreting cells in response to peptide pulsing than the negative controls ($P < 0.001$). The number of IFN-γ secreting cells measured by ELISpot assay, were approximately 11-fold higher than the controls (Fig. 7). When tested by intracellular cytokine staining for IFN-γ, a 30-fold increase in the number of $CD8^+CD62L^{low}$ IFN-γ secreting cells from *Lm*–LLO–PSA immunized mice was detected as compared to the control (4.22% for *Lm*–LLO–PSA vs. 0.15% for naïves) (Fig. 8).

Cytotoxicity (CTL) assay

In order to determine the functional activity of the T cells specific for PSA, splenocytes from immunized or naïve mice were tested in a CTL assay against cells pulsed with the $H2D^b$ PSA peptide [20]. Using fixed peptide concentration (1 μM), splenocytes from *Lm*–LLO–PSA immunized mice showed a maximum specific lysis of 60%, which was reduced in proportion to the E:T ratio (Fig. 9a). This outcome was dependent on the peptide concentration in the lysis assay with a maximum value reaching at a peptide concentration of only 0.1 μM (Fig. 9b). The SC_{50} value of the polyclonal population, which is a useful measure of average T cell avidity [27, 28] was approximately 1 pM. The results from this experiment also confirmed that immunization with *Lm*–LLO–PSA generated specific cytolytic T cells of high avidity capable of lysing cells that display the target antigen PSA.

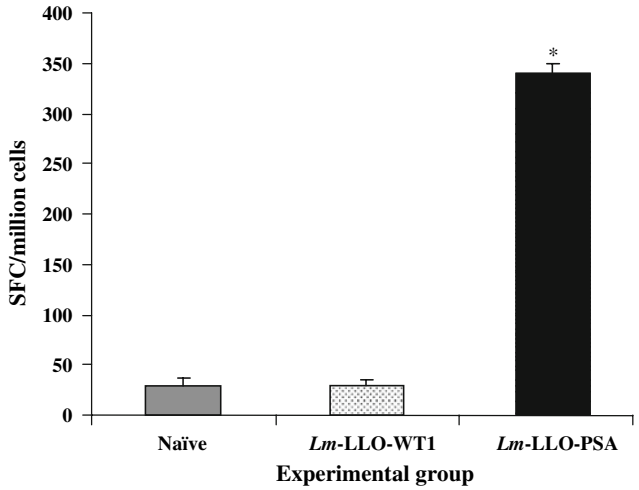

Fig. 7 IFN-γ secretion stimulated by *Lm*–LLO–PSA vaccination detected by ELISpot assay. Mice were immunized three times with 0.1 LD_{50} of *Lm*–LLO–PSA. Isolated splenocytes were prepared and incubated overnight in the presence of a 1 μM PSA specific $H2D^b$ peptide, HCIRNKSVIL. IFN-γ secretion by isolated cells was detected using the ELISpot kit from Mabtech and expressed as spot forming cells (SFC)/million cells. Splenocytes from *Lm*-LLO-WT1 immunized or naïve mice were used as negative controls. (* indicates statistical significance with the naïve groups with $P < 0.0001$)

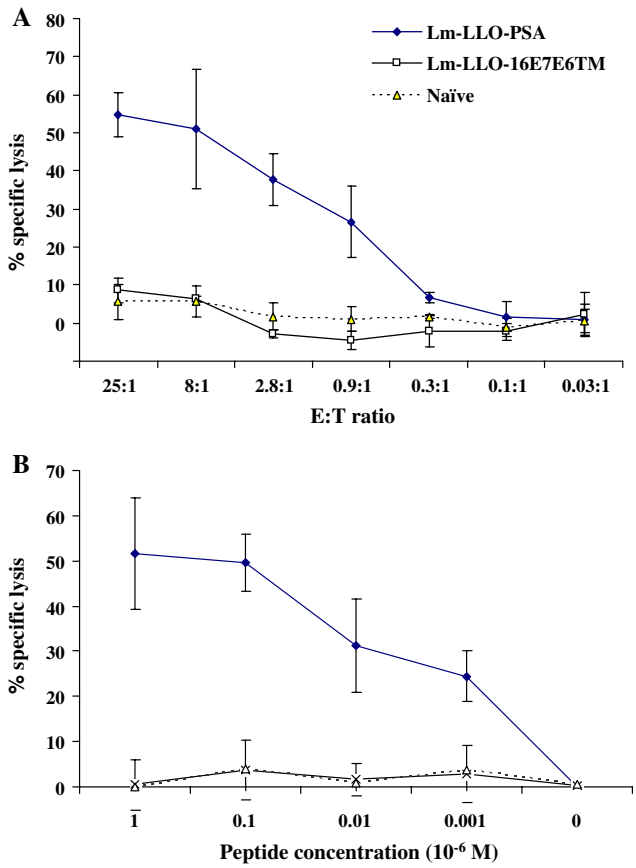

Fig. 9 PSA-specific cytotoxic responses in mice. Animals were immunized three times with 0.1 LD_{50} of *Lm*–LLO–PSA, *Lm*-LLO-HPV16E7E6 (as *Lm* negative control) or were left naïve. Isolated splenocytes were prepared and incubated with PSA expressing stimulator cells for 5 days. CTL assay was performed for 4 h by incubating effector cells (*E*) with target EL4 cells pulsed with PSA $H2D^b$ peptide: **a** at different E:T ratio/fixed peptide concentration (1 μM); or **b** at a fixed E:T ratio of 25:1 but different peptide concentrations

Comparison of the anti-tumor effects of *Lm*–LLO–PSA with other PSA based vaccine modalities

Finally to further evaluate *Lm*–LLO–PSA vaccine we compared its anti-tumor efficacy with two other PSA based vaccines that have been described in the literature i.e., a recombinant DNA and a vaccinia based vaccine. For this, the complete open reading frame of PSA was cloned into pCDNA3.1 under the control hCMV promoter (pAdv40.1). A similar DNA vaccine was previously described by Roos et al. [12] using a similar vector backbone i.e., pVax (Invitrogen). The difference between the two vector backbones (pCDNA3.1 and pVax) is only in their antibiotic selection marker. pAdv40.1 was injected i.m. as a mixture with GM-CSF/pCDNA, which has been used as an effective adjuvant for other DNA vaccines [29, 30]. Expression of PSA from

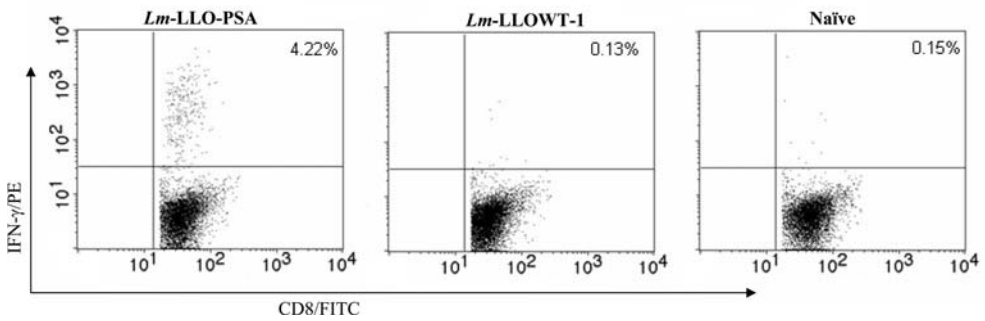

Fig. 8 IFN-γ production stimulated by *Lm*–LLO–PSA vaccination detected by intracellular staining. Mice were immunized three times with 0.1 LD_{50} of *Lm*–LLO–PSA. Isolated splenocytes were prepared and incubated for 5 h in the presence of a PSA specific $H2D^b$ peptide. IFN-γ production by activated $CD8^+$ T cells was determined as described in methods. Splenocytes from *Lm*-LLO-WT1 immunized or naïve mice were used as negative controls

PSA/pCDNA plasmid was confirmed by in vitro transfection into 293FT cell line (data not shown). Recombinant PSA-vaccinia has been previously described by Hudge et al. [31]. Mice were implanted with T-PSA23 tumor cells as described above and then immunized twice with each of the vaccines by the optimal dose and route of administration as cited in the literature [12, 32]. Immunization with PSA/vaccinia did not have any impact on the growth of T-PSA23 tumors as all naïve and PSA/vaccinia immunized mice developed tumors which reached a size of 2 cm within 7–8 weeks and they had to be sacrificed (Fig. 10b). However 1 out of 8 mice immunized with the PSA/DNA vaccine and 3 out of 8 mice vaccinated with *Lm*–LLO–PSA remained tumor free for over 90 days, at the time the study was terminated (Fig. 10a and the table insert). This experiment was repeated twice showing similar results. Thus, *Lm*–LLO–PSA was proven to be the most efficacious vaccine of the three PSA-vaccine modalities in regressing established PSA expressing tumors. It has to be noted that in the comparison studies, because of the limited availability of the other two vaccines, only two doses were administered. Therefore, *Lm*–LLO–PSA was shown to be less efficacious (complete tumor regression was observed in only three out of eight) than when injected three times (where seven out of eight tumors regressed, as seen in Fig. 4). This observation was confirmed in several other studies (data not shown).

Discussion

Despite a wide range of available treatments, pCa remains the second leading cause of cancer-related death among American men [1]. Recently, immunotherapeutic approaches which target specific tumor associated antigens have emerged as a potential alternative or complementary treatment for cancers. A number of prostate associated antigens have been identified in pCa, which have shown potential as immunotherapeutic targets [7]. These include Prostate Specific Antigen (PSA), Prostate Specific Membrane Antigen (PSMA), Prostatic Acid Phosphatase (PAP), Prostein, Prostate Specific G Protein coupled Receptor (PSGR), trp-p8 and Prostate Stem-Cell Antigen (PSCA) [4]. Although overexpressed in pCa, some of these antigens have also been detected in normal tissues. For instance, PSMA, one of the most studied prostate antigens, shows detectable traces in brain, liver and kidney [7]. Extra-prostatic presence of target antigens is an important factor when developing cancer vaccines, as immunizations against these proteins might trigger a pathologic autoimmune response. While being highly specific for prostatic epithelial cells in normal individuals, PSA is over-expressed in most cases of pCa, and therefore is widely used as a marker for its diagnosis and as an immunotherapeutic target to seek specific immune responses against cancer cells expressing it. These reasons led us to select PSA as a target for developing the

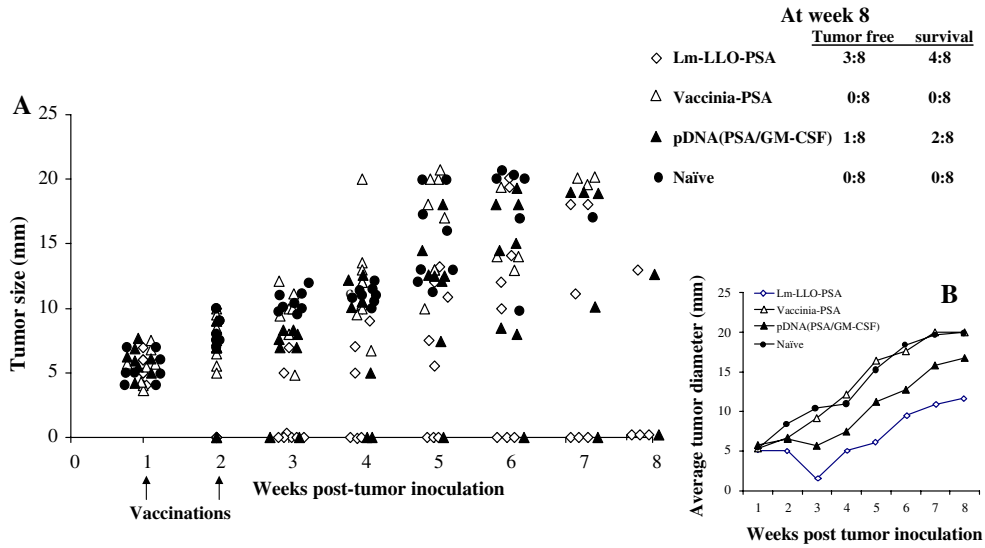

Fig. 10 Comparison of anti-tumor effects of *Lm*–LLO–PSA with other PSA based vaccines. Groups of 8 mice were inoculated s.c. with 5×10^6 T-PSA23 cells on day 0 and immunized two times with 1 week intervals, with *Lm*–LLO–PSA, vaccinia-PSA or pDNA (PSA+GM-CSF) or received no immunization (naïve). Tumors were monitored weekly using an electronic caliper and are expressed as the mean of two perpendicular diameters. Mice were sacrificed when the tumors reached a size of 20 mm in diameter. Results are depicted as tumor size from each individual mouse (**a**). The table shows the number of mice in each group, which were either tumor free or survived for 8 weeks post-tumor inoculation. **b** Average of tumor sizes in each group. When tumors reached a size of 20 mm, mice were sacrificed. They were given a value of 20 mm for making the average curve. This experiment was repeated two times showing similar results

first *Lm*-based therapeutic vaccine (*Lm*–LLO–PSA) against pCa. We showed that *Lm*–LLO–PSA could successfully express and secrete the fusion protein LLO-PSA, is stable after in vitro and in vivo growth and is readily taken up by macrophages in vitro in a similar way to that of the wild type *Lm* vaccine strain 10403 s.

In order to test the anti-tumor effects of *Lm*–LLO–PSA, we stably transfected a mouse prostate adenocarcinoma cell line (TRAMPC-1) with the complete gene of human PSA including its secretion signal sequence. These cells were shown to express and secrete the human PSA both in vitro and in vivo. In a therapeutic setting, using this model, we were able to show that *Lm*–LLO–PSA could cause regression of PSA-expressing tumors in more than 80% of cases. This effect lasted for at least 8 weeks. When sera from control or *Lm*–LLO–PSA immunized mice bearing T-PSA23 tumors of a diameter of 20 mm (on day 40 post-tumor inoculation) were tested by ELISA, significant levels of serum PSA were detected (~5 to 10 ng/ml). In contrast no PSA protein was detected in the sera from mice immunized with *Lm*–LLO–PSA, which had undergone tumor regression. This correlation is similar to that of human pCa as high serum PSA levels are used as a diagnostic measure for this type of cancer. It should be noted that PSA levels in the serum of *Lm*–LLO–PSA immunized mice undergoing tumor escape were comparable to those of the control mice with tumors. Thus, the tumor escape does not seem to be due to the loss of antigen (PSA) expression by the cells but other factors might be involved such as the presence of tumor infiltrating T-regulatory cells (suppressor Tregs) [33, 34]. We next looked at the different T cell populations in tumors to determine their impact in regression or progression of tumors. Since upon vaccination with *Lm*–LLO–PSA most tumors underwent complete regression, we injected the cells as a mixture with matrigel. First we found that immunization with *Lm*–LLO–PSA caused a significant increase in the number of PSA specific $CD8^+$ T cells in spleens and in the tumors as compared to control mice. Second, we showed that whereas in naïve tumors up to 25% of $CD4^+$ T cells display Treg phenotype ($CD4^+/CD25^+/FoxP3^+$), in mice immunized with *Lm*–LLO–PSA or the *Lm* control (*Lm*-LLO-E7), this number was reduced to almost a half. Both *Lm* constructs caused a decline in Treg numbers in the tumors. This is in accordance with previous published data [35], showing that in addition to their antigen specific effect, *Lm* based vaccines can cause a decrease in Treg allocation to tumors in a non-antigen specific manner.

Interestingly, although PSA is a secreted protein, this does not seem to impair the antigen presentation on the surface of T-PSA23 cells, which can be detected and eliminated by the immune system. This is encouraging, as it shows that immunization with recombinant *Lm* based vaccines is not only effective against intracellular antigens such as HPV-16 E7 [13] or cell surface bound proteins such as Her2/neu [15] but also can eliminate tumors expressing secreted antigens.

A major goal of immunization with cancer vaccines is to induce activation of antigen specific cells such as $CD8^+$ T cells, which can find and eliminate antigen presenting tumor cells [36]. The activated $CD8^+$ T cells release pro-inflammatory cytokines and cytolytic agents that attack and kill the tumor cells [37]. Vaccination with *Lm*-based vaccines preferentially stimulates this subset of T-cells [16]. Therefore we investigated the cellular responses elicited upon immunization with *Lm*–LLO–PSA by testing for IFN-γ secretion and cytotoxic activity in response to a PSA-derived peptide restricted to the MHC-class I molecule $H\text{-}2D^b$. High levels of IFN-γ secretion by splenocytes were detected by both ELISpot and intracellular cytokine staining with results being highly consistent and reproducible between the two assays. We also investigated the cytolytic function of the T-cells generated upon immunization with *Lm*–LLO–PSA by CTL assay and showed that these T cells are able to recognize and lyse peptide-pulsed target cells in vitro. In addition, the T cells generated upon vaccination appear to be of high avidity, with SC_{50} of about 1 pmole.

It is very encouraging that *Lm*–LLO–PSA raised strong and effective immune responses against PSA in our murine model; however these results were obtained from an animal species where the tumor antigen is foreign, whereas in humans, PSA is a self protein and might induce tolerance. Thus, the question still remains unanswered: will *Lm*–LLO–PSA be able to break the tolerance toward PSA in human? Previously, we have shown that *Lm* based vaccines are effective in this context. In transgenic mice for HPV16E7 or Her2/neu antigens, *Lm*-LLO-E7 and *Lm*-Her2/neu vaccines were able to overcome tolerance and cause regression of established solid tumors, respectively [38, 39]. Since there was no PSA transgenic mouse model available and mice lack intrinsic PSA or any PSA homolog, we were not able to test *Lm*–LLO–PSA vaccine for tolerance in a murine strain. As an alternative, male cynomolgus monkeys have shown to express a PSA protein with 89.7% homology with human PSA [10], making this species an attractive candidate for future evaluation of *Lm*–LLO–PSA immunogenicity as well as elucidation of any side effects that might arise upon vaccination with this self antigen. In any case, other investigators have shown that tolerance to PSA can be broken by repeated immunizations in humans [9, 40].

Anti-tumor effects of vaccines targeting PSA have also been shown in other preclinical studies, when the antigen was delivered by other vectors such as pDNA or vaccinia. In the present paper, we made a rough comparison of *Lm*–LLO–PSA with these other two PSA vaccine modalities. In an experimental setting where mice were primed and

boosted with the same vaccine, we found that when delivered by *Lm*, PSA was far more efficacious as an antigen than when delivered by the viral vector, vaccinia or naked DNA + adjuvant GM-CSF/pDNA. A pDNA vaccine based on PSA gene was previously reported by other investigators [12], where a combination of (PSA/IL-2) pDNA and GM-CSF was able to protect 80% of mice against challenge with PSA-expressing tumors. Although the therapeutic effects of the vaccine were not tested in those preclinical studies, in a phase I clinical trial conducted in hormone-refractory patients, PSA/pVax was reported to be safe and elicited some cellular responses toward PSA [41]; while its efficacy is still to be determined. Overall, DNA vaccines have shown to be a safe alternative to live vaccine vectors although their efficacy in human is low due to their insufficient uptake by antigen presenting cells (APCs). To face this challenge, different delivery methods are being investigated; for instance when applied by intradermal electroporation in mice, PSA/pVax showed significantly higher gene expression and enhanced cellular responses toward PSA than when injected as naked DNA [29], although for human use, this procedure still has to be further developed. In this context, the advantage of *Listeria* as a vaccine vector lays in its specific ability to activate and deliver antigens into the intracellular environment of professional APCs such as macrophages and dendritic cells, resulting in strong cellular immune responses against these antigens, while raising minimum humoral responses against the bacterium itself [42]. *Lm* also has advantage over live viral vaccine vectors such as vaccinia and adenovirus which upon primary vaccination result in strong humoral responses against the vector, reducing the chances of benefiting from booster doses when using the same vector [43]. This might be the reason behind the low efficacy we observed with PSA/vaccinia when administered twice. Therefore, sequential and complex prime/boost regimens had to be set up for immunization with recombinant PSA-vaccinia/fowlpox virus vaccines to obtain maximum efficacy [43]. Also it has to be noted that both PSA/pDNA and PSA/vaccinia express the secreted form of PSA because both constructs retain the intrinsic PSA secretion signal sequence. Therefore, upon expression by either vaccine vector, PSA will be preferentially secreted out of the host cells. In contrast, although LLO-PSA is secreted out of *Lm*–LLO–PSA, it is trapped in the host cytoplasm, and immediately directed into the protoesome degradation pathway [22], resulting in a significantly improved MHCI antigen presentation. Thus, this small difference between the designs of the three vaccine systems might affect the final destination of the target antigen and as a result the quality of the immune response that is generated against it, which in our case is shown by the superior anti-tumor effects of *Lm*–LLO–PSA. Another very promising approach in prostate cancer immunotherapy is vaccination with autologous dendritic cells which are loaded with peptides, recombinant proteins, tumor lysates or messenger RNA [9, 44, 45]. An immunotherapy product consisting of APCs, loaded ex vivo with a fusion of PAP and granulocyte-macrophage colony-stimulating factor (Sipuleucel-T), demonstrated promising results when tested in a phase III, placebo-controlled trial [46]. Although the use of autologous DCs for antigen presentation seems to be efficacious in human, the production of these individualized vaccines is extremely costly. In contrast, *Lm*-based vaccines are not patient-specific; the production is easy and of low-cost and can be used to treat any cancer patient with a competent immune system.

Taken together, the data presented in this paper indicate that a recombinant *Lm*, which can express and secrete the human PSA protein fused to LLO, can generate an effective and durable anti-tumor response in mice. This effect is associated with strong and specific immune responses toward PSA. These encouraging results provide the scientific rationale for further investigation of the vaccine as a new immunotherapeutic agent against pCa in human.

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