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Construction and Assessment of a Novel Vaccine Targeting Hepatocellular Carcinoma

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IMMUNOTHERAPY is a promising and highly effective method of targeting hepatocellular carcinoma (HCC) cells and improving patient outcomes. There is a continuing need for the development and refinement of current vaccine vehicles to safely target tumors while stimulating robust cell-mediated immune responses. The facultative intracellular bacterium *Listeria monocytogenes* has proven to be an effective vehicle for the design of cancer vaccines that stimulate potent and long $CD8^+$ T-cell responses. Critical facets of its effectiveness relate to the ability of gaining access to the cytosol of infected host cells and delivering tumor-associated antigens. Here we describe the development and testing of *L. monocytogenes* Δ*prsA2* Δ*htrA* strains as effective vaccine vehicles for the safe delivery of HCC antigens. Recombinant *L. monocytogenes* Δ*prsA2* Δ*htrA* was engineered to express α-fetoprotein antigens designed to elicit immunity against HCC cells, and tested in a subcutaneous mouse model together with the previously developed *L. monocytogenes ΔactA prfA** strain. The results suggest that *L. monocytogenes* Δ*prsA2* Δ*htrA* strains may represent a highly attenuated yet effective vaccine vector capable of stimulating immunity against HCC cells *in vivo*.

Keywords: Hepatocellular carcinoma, Immunotherapy, *Listeria monocytogenes,* Tumors, Vaccine vectors.

Introduction

Hepatocellular carcinoma (HCC) is the second leading cause of cancer related deaths worldwide. It is a major threat to the public heath as 800,000 patients are dying from this disease every year [1]. According to the Barcelona clinic liver cancer classification system, localized tumors could be treated by resection and liver transplantation. Cryoablation, percutaneous ethanol injection and transarterial chemoembolization are local therapies used to block blood supply and induce subsequent tumor necrosis; however, only 20% of patients are eligible [2]. Sorafenib is the only systemic tyrosine kinase inhibitor approved by FDA for treating patients with advanced HCC [1]. HCC cells express elevated levels of multidrug resistant proteins, which make it resistant to treatment options. In addition, HCC patients have impaired metabolizing properties that prevent them from tolerating the hepatotoxic effects of systemic therapies [3]. Therefore, immunotherapeutic strategies represent an attractive alternative that could alter the immunosuppressive microenvironment of HCC [4]. The immune response against HCC is mediated mainly through cellular immunity [5]. Transformed cancer cells have tumor associated antigens (TAA) that are recognized as nonself. Antigen presenting cells (APCs) uptake, process

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and present the TAA on major histocompatability complex (MHC) class I to activate effector T cells. CD8 cytotoxic T lymphocytes carry out immunologic surveillance; they recognize antigens on HCC cells, and kill them by direct lysis [6].

Live attenuated bacteria, including the promising vaccine vector *Listeria monocytogenes* (*L.monocytogenes*), have emerged as a possible immunotherapeutic option that can break the tolerance and elicit a robust immune response [7]. *L.monocytogenes* strains can be engineered to secrete alpha fetoprotein (AFP), a HCC antigen that is abundantly expressed in HCC, and has been consistently associated with increased tumor proliferation and poor prognosis [8]. As an intracellular parasite, *L.monocytogenes* has direct access to the cytoplasm of APCs where it can grow, multiply and secrete the tumor antigens which stimulate the MHC class I presentations pathways; and induce CD8 antigen specific T cell response [9]. To ensure safety, highly attenuated strains of *L.monocytogenes*, that lack one or more of the virulence genes, are often conducted. The secretion chaperones of *L.monocytogenes* are considered among the main virulence genes because they are essential for bacterial infection of host cells [10]. Interestingly, *L.monocytogenes ΔactA prfA** attenuated strain, that cannot spread from cell to cell, was found to elicit a robust and specific immune response [11], [12].

We hypothesized that super attenuated *L.monocytogenes ΔprsA2 ΔhtrA* strain would be able to elicit an immune response against tumors, and would represent a safer vaccine strain than the previously tested ones due to absence of the two secretion chaperones. In this study, we construct recombinant *L.monocytogenes ΔprsA2 ΔhtrA* and *ΔactA prfA** strains that express AFP antigens; investigate the functionality, safety and efficacy of it against HCC in prophylactic mouse models, and finally compare the extent of tumor regression against less attenuated but clinically tested *ΔactA prfA** vaccine vectors.

Experimental: Materials and Methods

Bacterial strains, plasmids, and culture conditions

All bacterial strains and plasmids used in this study are listed in Table 1. *L. monocytogenes* NF-L100 strain was used as wild type (WT) control, and NF-L974 (*ΔactA prfA**) and NF-L1633 (*ΔprsA2 ΔhtrA*) strains were used as the parent strains for the construction of recombinant vaccine strains. *Escherichia coli* One Shot TOP10 and SM10 (Invitrogen Corp., Carlsbad, CA) were used as host strains for maintenance and propagation of recombinant plasmids. *L. monocytogenes* and *E. coli* strains were grown with agitation overnight at 37°C in brain heart infusion (BHI) media (Difco Laboratories, Detroit, MI) and Luria broth (LB) (Invitrogen Corp., Carlsbad, CA) unless specifically stated otherwise. Maintenance of the integration of the Myc-tagged antigen expression vector plasmid pPL6-PA was selected for using 25 µg/ml of chloramphenicol in *E. coli* and 7.5 µg/ml in *L. monocytogenes*. *E. coli* containing the tumor antigen expression vector (pUC18) was maintained in Luria broth with 50 µg/ml carbenicillin. Streptomycin 200 µg/ml was used in selection of *L. monocytogenes* following bacterial conjugation and isolation from tissue organs of infected mice.

Mouse strain and cell lines

C57BL/6 (#000664) female mice 8 to 10 weeks old were purchased from the Jackson Laboratory (Bar Harbor ME). Hepa1–6, a HCC cell line derived from C57BL/6 mice and J774A.1 mouse macrophage cell line were purchased from the American Type Culture Collection (ATCC). Hepa1–6 and J774A.1 cell lines were maintained in Dulbecco's modified Eagle's medium with 10% heat-inactivated fetal bovine serum (Gibco, Carlsbad, CA, USA) at 37°C in 5% CO2 incubator.

Selection of tumor antigens and codon optimization

To assess the efficacy of *L. monocytogenes ΔprsA2 ΔhtrA* strains in stimulating protective immune responses against HCC, murine AFP which is abundantly expressed on HCC cells but not normal adult mouse liver was selected as the test tumor antigen. Antigenic fragments of alpha fetoprotein $(AFP)_{137-145}$, $AFP_{158-166}$ $AFP_{325-334}$ and $AFP_{542-550}$ represent highly immunogenic epitopes that can elicit a robust immune responses and protect against HCC. The aforementioned antigenic epitopes were extracted using CLC sequence viewer 7.8.1, and dissected into two fragments: AFP1 containing the two epitopes $AFP_{137-145}$ and $AFP_{158-166}$, and AFP2 containing $AFP_{325-334}$ and $AFP_{542-550}$ [13], [14]. The sequences encoding the antigens were sent to GenScript (860 Centennial Ave, Piscataway, NJ 08854, USA) to be codon optimized for maximal protein secretion in *L. monocytogenes*. The codon adaptation index (CAI), which is the extent of the fragments expression with respect to the highly expressed genes, was modified to 0.94, with a CAI of > 0.8 being regarded as good in terms of high level protein expression in *L. monocytogenes*. In addition, the GC content, which should range from 30 to 50%, was modified to be 34.45 to potentially prolong the half-life of mRNA in *L. monocytogenes*. Potential mRNA stem loop structures were abrogated to increase ribosome binding and the stability of the mRNA. Finally, BamHI restriction sites were added to the ends of each fragment to facilitate cloning initially into pUC18 vectors.

TABLE 1. Bacterial strains and plasmids used in the study.

Strain	Description	Reference
NF-L100	Wild-type 10403S *L.monocytogenes* parent strain	[40]
NF-L974	ΔactA prfA* act A deletion in *L.monocytogenes* 104035 + modified allele of *prfA* that results in the expression of a moderately activated PrfA while retaining a cell-to-cell spread defect that confers attenuation. PrfA is a transcriptional activator that regulates the expression of many of the secreted and surface associated proteins that contribute to *L. monocytogenes* host cell invasion and intracellular growth. PrfA becomes activated upon entry of *L. monocytogenes* into host cells, and PrfA activation results in a dramatic increase in *L. monocytogenes* virulence factor. This mutant strain was found by two separate groups to elicit a robust and specific immune response.	[11], [12], [24]
NF-L1633	ΔprsA2 ΔhtrA htrA deletion in *L. monocytogenes*104035 + transduced with erm tagged ΔprsA2 PrsA2 is a secreted post-translocation chaperone with both foldase and cis, trans peptidyl proline isomerase (PPIase) activity that contributes to the folding of secreted virulence factors. PrsA2 is located at the bacterial membrane – cell wall interface, and mutants lacking PrsA2 are highly attenuated in mouse infection models. Working in concert with PrsA2 is a secreted chaperone protease known as HtrA; it contributes to protein folding as well as the degradation of misfolded proteins at the bacterial cell surface. Both PrsA2 and HtrA are important for bacterial infection of host cells, and bacteria that lack both chaperones are extremely attenuated.	[40], [22]
NF-E1699	E. coli SM10 (conjugation strain) containing pPL6-PA-Myc plasmid	[24]
NF-L4364	ΔprsA2 ΔhtrA *L. monocytogenes* NF-L1633 strain containing pPL6-PA-Myc plasmid	This work
NF-L4365	ΔprsA2 ΔhtrA *L. monocytogenes* NF-L1633 strain containing pPL6-PA-AFP1-Myc plasmid	This work
NF-L4366	ΔprsA2 ΔhtrA *L. monocytogenes* NF-L1633 strain containing pPL6-PA-AFP2-Myc plasmid	This work
NF-L4368	ΔactA prfA* *L. monocytogenes* NF-L974 strain containing pPL6-PA-Myc plasmid	This work
NF-L4369	ΔactA prfA* *L. monocytogenes* NF-L974 strain containing pPL6-PA-AFP1-Myc plasmid	This work
NF-L4370	ΔactA prfA* *L. monocytogenes* NF-L974 strain containing pPL6-PA-AFP2-Myc plasmid	This work
Plasmids	Description	Reference
pPL6-PA-Myc	Plasmid pPL6-PA-myc (NF-E1699) is the main plasmid used throughout the study; it is a shuttle vector that stably integrates to a single locus inside Lm genome through a specific phage attachment site (PSA), and it could be easily selected through its endogenous chloramphenicol (Cam) resistance. The plasmid has the promoter, ribosomal binding site and the highly secretory signal peptide of the hly gene in which different antigens could be secreted under its influence. The plasmid also has multiple cloning sites (MCS) to facilitate cloning of different antigens and a C-myc tag which served as a marker to detect protein expression and secretion. Plasmid pPL6-PA-myc has also protective antigen (PA) from Bacillus Anthrax that has been observed to over-stimulate the secretion of cloned antigens.	[24]
pPL6-PA-AFP1-Myc	pPL6 contains HCC TAA alpha fetoprotein 1, which encodes $AFP_{137-145}$ and $AFP_{158-166}$ epitopes	This work
pPL6-PA-AFP2-Myc	pPL6 contains HCC TAA alpha fetoprotein 1, which encodes $AFP_{325-334}$ and $AFP_{542-550}$ epitopes	This work

Construction of L. monocytogenes attenuated vaccine vectors expressing AFP antigens

The codon optimized genes encoding AFP1 and AFP2 were subcloned from pUC18 plasmids by restriction digestion with BamHI followed by gel purification of the gene fragments using the Qiagen purification kit (Cat No. 28104, Qiagen company, Germantown, Maryland, USA). Purified gene fragments were inserted into the pPL6-PA-Myc expression plasmid previously digested with BamHI, the resultant plasmids were designated pPL6-PA-AFP1-Myc and pPL6-PA-AFP2-Myc (Table 1). Plasmids were maintained in *E. coli* TOP10 cells for propagation [15], and electroporated into *E. coli* SM10 cells [16] for transfer into *L. monocytogenes* NF-L1633 (*ΔprsA2 ΔhtrA*) and NF-L974 (*ΔactA prfA**) strains by conjugation [17] with transconjugants selected on BHI plates containing 7.5 µg/ml of chloramphenicol/ml and 200 µg/ml of streptomycin. All genes and plasmids were verified by DNA sequencing performed at the UIC Research Resources Center Core Genomics Facility. Sequencing primers used for verification are listed in Table 2.

Generation of protein extracts and Western blot analysis

Secreted proteins were isolated from bacterial culture supernatants and bacterial surface-associated fractions were isolated from whole bacterial cells as previously described with minor modifications [18], [19]. In brief, 40 ml cultures of *L. monocytogenes* strains NF-L4368 (*ΔactA prfA** PA-Myc) and NF-L4369 (*ΔactA prfA** PA-AFP1-Myc) were grown to an OD_{600} of 0.8 in BHI broth at 37°C with shaking, and 40 ml cultures of strains NF-L4364 (*ΔprsA2 ΔhtrA* PA-Myc), NF-L4365 (*ΔprsA2 ΔhtrA* PA-AFP1-Myc), and NF-L4366 (*ΔprsA2 ΔhtrA* PA-AFP2-Myc) were grown to an OD_{600} of 0.6 in fresh LB containing 25 mM glucose-1-phosphate (G-1-P), 0.2% activated charcoal, and 50mM morpholino propane sulfonic acid (MOPS) at pH7.3, 37°C without shaking [20], [21]. All cultures were normalized by adjusting cultures to OD_{600} of 0.5 prior to protein fractionation. Proteins present in the culture supernatants were precipitated with 10% trichloroacetic acid (TCA) (Fisher Scientific) at -20°C for at least 12 hours, followed by centrifugation and two washes of the protein pellets with ice-cold acetone. Pellets were resuspended in 200 µl of 2X SDS boiling buffer (Bio-Rad) and boiled for 10 minutes at 100°C. The protein samples were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) using 4-15% ammonium persulfate gradient gels and transferred onto polyvinylidene difluoride (PVDF) membranes followed by blocking by soaking in 5% dried milk. Secreted proteins PA-AFP1 and PA-AFP2 were detected by incubation with commercial anti-Myc antibody at a dilution of 1:500, followed by incubation in anti-mouse secondary antibody conjugated to alkaline phosphatase at a 1:2000 dilution. Bands were then visualized colorimetrically with the addition of 10 ml of a BCIP/NBT Plus solution (SouthernBiotech, Birmingham, AL).

Growth of bacterial vaccine vectors in broth and in tissue culture cells

Bacterial growth was measured in BHI broth beginning with a 1:20 dilution of overnight cultures into fresh BHI. Growth was measured each hour by determining the absorbance at an optical density of 600 nm. Bacterial intracellular growth assays in mouse macrophage-like cells (J774A.1) were performed as previously described [22] with minor modifications. The bacterial strains NF-L100, NF-L4364, NF-L4365, NF-L4366, NF-L4368 and NF-L4369 were grown in BHI without shaking at 37°C overnight; and monolayers of the mammalian cells were grown on glass coverslips to confluence and infected with the bacterial cultures with a multiplicity of infection (MOI) of 5:1. One hour post-infection, monolayers were washed three times in Dulbecco's phosphate-buffered saline (DPBS) (Cellgro Mediatech Inc., Herndon, Virginia, USA), and fresh medium was added, followed by 5 µg/ml of gentamicin to kill extracellular bacteria. At 3, 5 and 7 hours post-infection, coverslips were removed and lysed in 2 ml of sterile H_2O with vigorous vortexing to release intracellular bacteria. Lysates were then spread onto LB agar plates, incubated at 37°C overnight, and bacterial colony forming units (CFU) were enumerated on the following day.

TABLE 2. Oligonucleotides used in the study.

Primer	Sequence from 5' to 3'	Reference
Hly Forward 1	GAT AAT CAA AACTAT CGT TGC	[24]
Hly Forward 2	GCG TTT CAT CTT TAG AAG CG	This work
Hly Forward 3	GGC GCC AAT CGC ATT AAA TGC	This work
Hly Reverse	TTT GCT TCA GTT TGT TGC GC	[24]
Myc Reverse	CTA AAG ATC TTC TTC AGA AAT AAG	[24]

Detecting in-vivo safety of L. monocytogenes strains

C57BL/6 mice were randomly allocated into six groups (n=5), the animals received two immunizations, one week apart, via retro-orbital injection according to the following scheme: Control group: received 0.1 ml of phosphate buffered saline (PBS). Groups NF-L4364, NF-L4365 and NF-L4366 received $1x10^8$ CFUs (0.1 LD50, [23] of *ΔprsA2 ΔhtrA* strain containing plasmids pPL6 -PA-myc, pPL6 -PA-AFP1-myc and pPL6 -PA-AFP2-myc respectively in a total volume of 0.1 ml PBS. Groups NF-L4368 and NF-L4369 received $1x10^7$ CFUs (0.1 LD50, [24] of *ΔactA prfA** strain containing plasmids pPL6-PA-myc and pPL6 -PA-AFP1-myc respectively in a total volume of 0.1 ml PBS. Three days following the last immunization dose, mice were anesthetized by intraperitoneal (IP) injection of 100 mg/kg Ketamine and 8 mg/kg Xylazine. Blood samples were withdrawn from the retro-orbital vein, and serum was separated by centrifugation [25]. The serum samples were sent to the UIC diagnostic laboratory for the evaluation of alanine amino-transferase (ALT) and aspartate amino-transferase (AST) tests of liver function. Half of the animals were euthanized by cervical dislocation, and then livers and spleens were aseptically harvested. Organs were homogenized in 5 ml sterile H2O using a tissue homogenizer and serial dilutions were plated onto BHI plates supplemented with 200 ug/ml streptomycin for CFU enumeration [26].

Determination of of body weights and survival rate for mice developed subcutaneous tumors

Subcutaneous tumors were induced in other sets of C57BL/6 mice (n=5) through inoculation of $5x10^5$ sorted Hepa1-6 cells in the left flank region. When the average tumors volume reached 150 mm^3, three immunizations of 10^8 CFUs of ΔprsA2 ΔhtrA strains or 10^7 CFUs of ΔactA prfA*strains or PBS were injected intravenously one week apart via the retro orbital vein. The body weights were recorded weekly for one month by a digital balance, and the survival rate was monitored on daily bases and calculated at the end of the study using Kaplan–Meier estimator.

Mouse immunization and HCC challenge using Hepa1 –6 cells

C57BL/6 mice were randomly allocated into six groups (n=10) and doses of *L.monocytogenes* vaccine strains were injected IP. The immunizations were repeated at the indicated intervals with the following concentrations: $1x10^8$ CFU for NF-L4364, NF-L4365, NF-L4366 strains and $1x10^7$ CFU for NF-L4368, NF-L4369 in a total volume of 0.1 ml PBS, and 0.1 ml of PBS was injected in the control group. Ten days after the second immunization, mice were inoculated subcutaneously (SC) following shaving their back hair in the left inguinal region with $5x10^6$ Hepa1–6 cells. After 10 days, tumors become visible and their volumes were measured every three days using a caliper; tumor volumes (V) were calculated with the equation $V= (L X W^2) X 0.5$ [27] where L and W represent the length and width of the tumor. The mice were sacrificed when the average tumor diameter reached 20 mm.

Isolation of splenocytes, cell surface staining and flow cytometry

At day 33 of the experiment, half of the animals in each group were euthanized as previously mentioned. Tumors and spleens were aseptically isolated, and the weight of each organ was recorded. Spleens were placed in a tube containing ice chilled 1X PBS. Splenocytes were harvested by teasing the spleens apart with the plunger of 5ml syringe, and the splenocytes suspensions were filtered through cell strainers (BD Falcon, San Jose, CA, USA), and digested with red blood cells (RBCs) lysis buffer (ebioscience, # 00-4333-57) to obtain a single-cell suspension, and eliminate RBCs [28]. The splenocytes were then centrifuged and resuspended in flow cytometry staining buffer (ebioscience, # 00-4222-26). For blocking of Fc receptors, splenocytes were incubated with TruStain fcX™ anti-mouse CD16/32 (Biolegend, #101319) for 10 minutes on ice. Then cells were stained with FITC anti-mouse CD8a (Biolegend, #100705) and Pacific Blue™ anti-mouse CD3 (Biolegend, #100213) antibodies at optimum concentrations; and incubated on ice for 30 minutes in the dark. Samples were sent to UIC Flow Cytometry facility for detection and quantification of $CD8^+$ $CD3^+$ cells by Gallios flow cytometer.

Statistical analyses

Statistical analyses were performed using GraphPad software version 2.0. Where appropriate, a one-way or two-way analysis of variance followed by Dunnett or Tukey's multiple comparison tests were used to identify statistical significance differences. A *P* value of ≤ 0.01 was considered significant.

Ethics statement

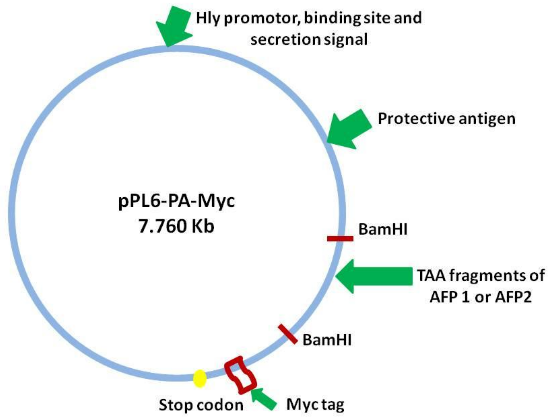
All experiments used in the study were approved from the ethics committee of Faculty of Pharmacy Cairo University #PT126 and the institutional review board of University of Illinois in Chicago #15-126-08. Animals were treated in accordance with Guide for the Care and Use of Laboratory Animals (8^{th} edition, National Academies Press).

Results

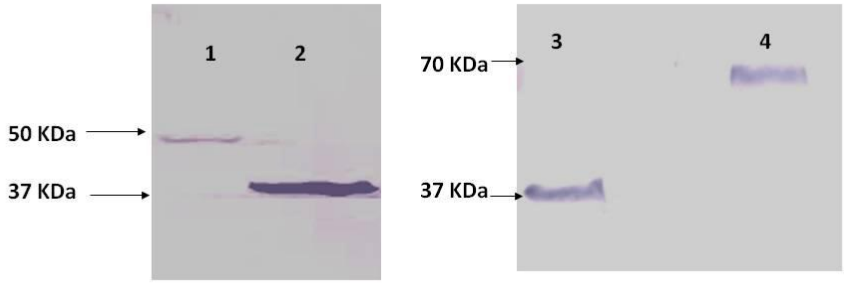
Construction of live attenuated L. monocytogenes strains secreting AFP

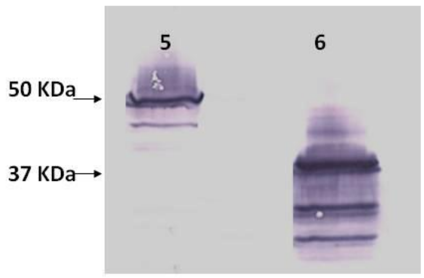
Gene fragments encoding predicted AFP immunodominant epitopes were codon optimized for expression in *L. monocytogenes* using a commercial vendor and these gene fragments were cloned into the pPL6-PA-Myc expression vector (Fig.1A and Table 3). Plasmid pPL6-PA-Myc features the promoter, the ribosomal binding site and the N-terminal secretion signal peptide derived from *hly* coding sequences to facilitate the secretion of foreign antigens. The expression vector includes an in-frame Myc tag thus enabling the detection of fused antigen protein expression and secretion. Additionally, pPL6-PA-Myc is designed for the generation of protein fusions to a portion of the Protective Antigen (PA) of *Bacillus anthracis*. Expression and secretion of the PA-AFP antigen fusions was confirmed for *L. monocytogenes ΔprsA2 ΔhtrA* strains as well as for *L. monocytogenes ΔactA prfA** strains; both antigen fusions were easily detectable in *L. monocytogenes* supernatant fractions and analyzed by western blotting (Fig. 1B).

1A



1B





Lane number	Strains and secreted proteins
1	NF-L4365 ΔprsA2 ΔhtrA PA AFP1
2	NF-L4364 ΔprsA2 ΔhtrA PA
3	NF-L4364 ΔprsA2 ΔhtrA PA
4	NF-L4366 ΔprsA2 ΔhtrA PA AFP2
5	NF-L4369 ΔactA prfA* PA AFP1
6	NF-L4368 ΔactA prfA* PA

Fig.1. Construction and characterization of recombinant *L. monocytogenes* vaccine strains. A) The cartoon map of pPL6-PA-Myc plasmid has been generated upon sequencing. By using the BamHI restriction site, the tumor associated antigens AFP1 or AFP2 were inserted downstream of the *hly* promoter, in frame with the secretion signal sequence of *hly*, and upstream of the Myc tag which served as a marker to detect protein expression and secretion. B) All resulting plasmids containing the TAA genes were individually transformed into SM10, and then integrated into the chromosome of attenuated *L.monocytogenes* strains through conjugation. Expression and secretion of PA-AFP1/AFP2 by recombinant *L.monocytogenes* strains were demonstrated in cell culture supernatants, and then detected by anti-Myc antibody.

TABLE 3. The amino acid sequence of tumor associated antigens AFP1 and AFP2.

Fragment	Amino Acid Sequence
AFP1	G S L S N K Y G L S G C C S Q S G V E R H Q C L L A R K K T A P A S V P P F Q F P E P A E S C K A H E E N R A V F M N R F I Y E V S R R N P F M Y A P A I L S L A A Q Y D K V V L A C C K A D N K E E C F Q T K R A S I A K E L R E G S M L N E H V C S V I R K G S
AFP2	I Q L P M I Q L G F C I I H A E N G V K P E G L S L N P S Q F L G D R N F A Q F S S E E K I M F M A S F L H E Y S R T H P N L P V S V I L R I A K T Y Q E I L E K C S Q S G N L P G C Q D N L E E E L Q K H I E E S Q A L S K Q S C A L Y Q T L G D Y K L Q N L F L I G Y T R K A P Q L T S A E L I D L T G K M V S I A S T C C Q L S E E K W S G C G E G M A D I F I G H L C I R N E A S P V N S G I S H C C N S S Y S N R R L C I T S F L R D E T Y A P P P F S E D K F I F H K D L C Q A Q G K A L Q T M K Q E L L I N L V K Q K P E L T E E Q L A A V T A D F S G L L E K C C K A Q D Q E V C F T E E G P R I

Characterization of L. monocytogenes recombinant vaccine growth

We first compared the growth of the recombinant strains with the parent and WT strains in broth culture; and there were no any significant differences. This shows that genetic modification and the secretion of tumor antigens did not impact the growth of mutant strains (Fig. 2A). Growth of the recombinant *L. monocytogenes* strains expressing tumor antigen was next assessed in tissue culture cells. Bacterial replication within infected J774 macrophage-like cells showed modest levels of inhibition for *L. monocytogenes* Δ*actA prfA** strains expressing tumor antigen in comparison to wild type bacteria (Figure 2B). *L. monocytogenes* Δ*prsA2* Δ*htrA* expressing PA alone was unable to replicate within infected cells and exhibited a modest decline in bacterial numbers (Fig. 2C). Interestingly, strains expressing the PA-AFP fusion proteins exhibited even greater defects, with bacterial numbers rapidly declining with increasing time post-infection (Fig. 2C). The growth defect observed for *L. monocytogenes* Δ*prsA2* Δ*htrA* strains expressing PA-AFP tumor antigen fusions within infected tissue culture cells suggested that these strains should pose little risk for bacterial infection of mice.

L. monocytogenes recombinant vaccines demonstrate safety in vivo

We thus compared the number of recoverable bacterial CFUs from target organs at three days post-infection for mice immunized with *L. monocytogenes* Δ*prsA2* Δ*htrA* strains and the Δ*actA prfA** mutant. Mice infected with Δ*prsA2* Δ*htrA* strains (NF-L4364 and NF-L4367) had no detectable CFUs recovered from either liver or spleen (Fig. 3A). In contrast, approximately half of the mice infected with Δ*actA prfA** strains exhibited detectable bacterial burdens within the liver while no bacteria were recovered from the spleen (Fig. 3B). Consistent with the reduced to undetectable bacterial burdens within the liver and spleen, mice immunized with Δ*prsA2* Δ*htrA* strains exhibited normal liver function as measured by serum AST and ALT levels (Fig. 3C).

Effect on body weight and survival rate for mice with subcutaneous tumor

Moreover, immunization with *L. monocytogenes* strains significantly prevented the decrease of body weight that happened in the control mice by 33%; and the *ΔprsA2 ΔhtrA* strains showed slightly higher protection than *ΔactA prfA** strains (Fig. 3D). Finally, mice immunized with *ΔprsA2 ΔhtrA* and *ΔactA prfA** strains exhibited high survival rates of 100% and 80% while the control ones exhibited only 40% (Fig. 3E). The results, collectively, confirm the safety of Δ*prsA2* Δ*htrA* strains *in vivo,* which make it a promising vaccine candidate.

L. monocytogenes Δ*prsA2* Δ*htrA strains stimulate tumor regression in mouse models of HCC*

To assess the ability of *L. monocytogenes* Δ*prsA2* Δ*htrA* recombinant vaccine strains to elicit protection against HCC tumors, mice were vaccinated twice with *L. monocytogenes* Δ*prsA2* Δ*htrA* or Δ*actA prfA** strains expressing PA-AFP fusions prior to sub-cutaneous inoculation of Hepa1-6 HCC cells (Fig. 4A). Ten days later after the second vaccination, Hepa1-6 HCC tumor cells were inoculated into the left inguinal region and subsequent mouse tumor volumes were monitored and compared. While all animals eventually clear the HCC tumors in this model, mice vaccinated with the *L. monocytogenes* Δ*prsA2* Δ*htrA* pPL6-PA-AFP1-Myc strain (NF-L4365) exhibited the most rapid elimination of tumor burdens (Figure

2A

2B

2C

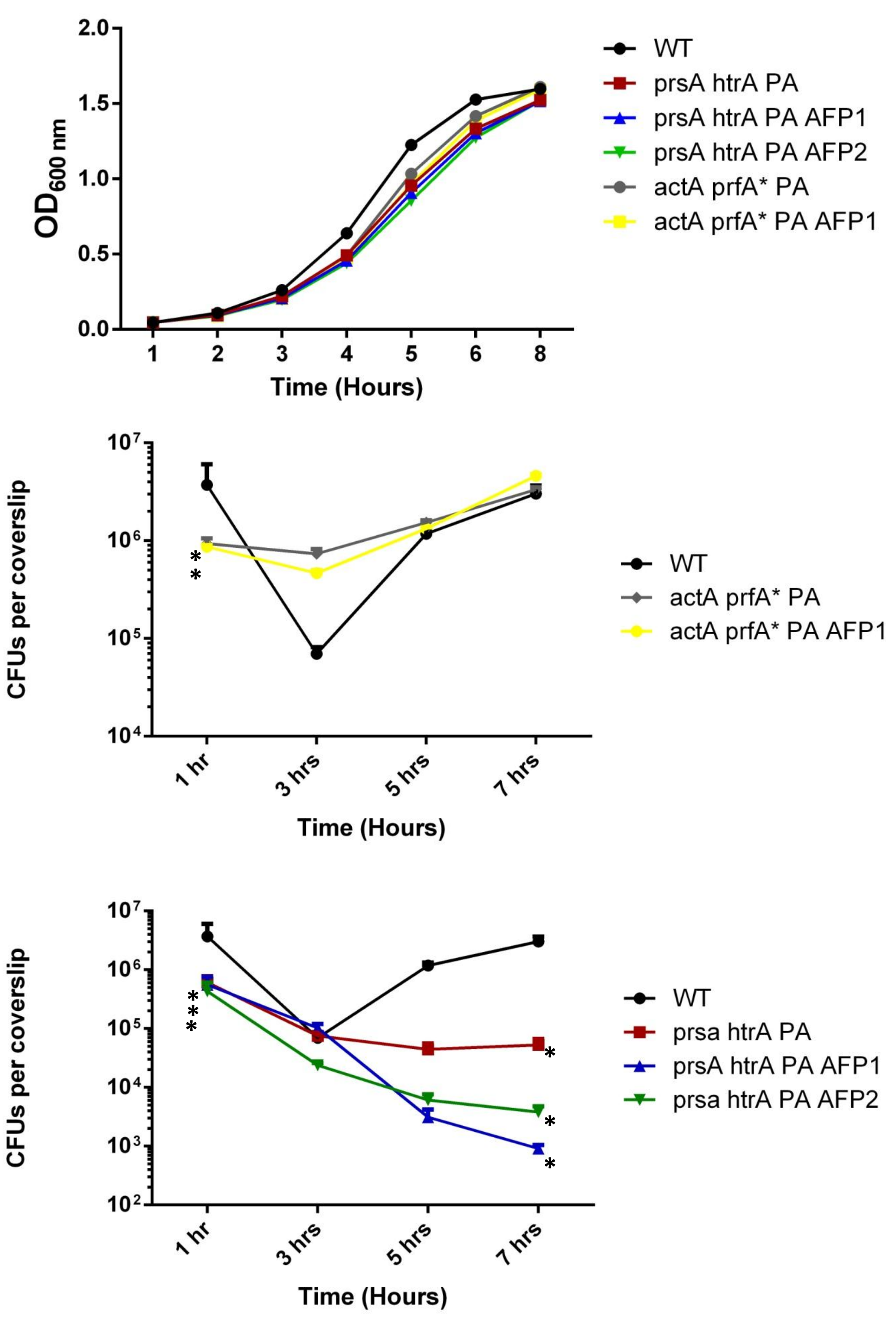
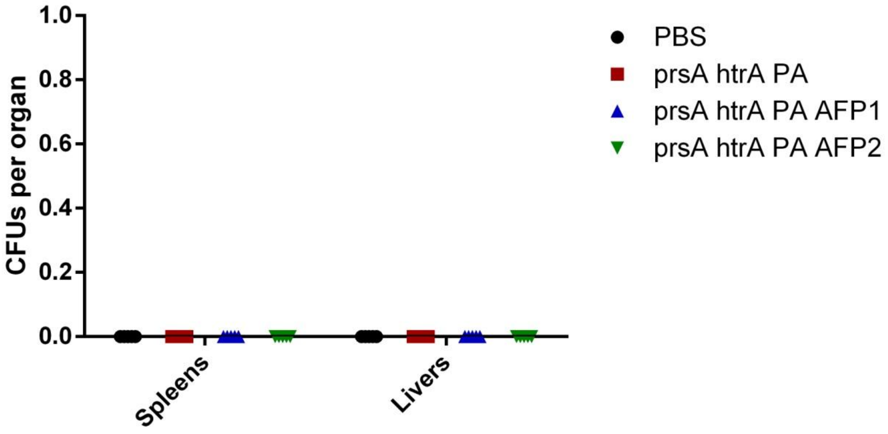


Fig. 2. Characterization of *ΔprsA2 ΔhtrA* and *ΔactA prfA* L.monocytogenes* recombinant vaccine strains. A) Bacterial growth in BHI broth at 37°C was determined via measuring the optical density at 600 nm at the indicated time points in the graph. The data shown are from two independent experiments. Data was analyzed by one-way analysis of variance followed by Dunnett test for multiple comparisons at *P* value of < 0.01, and no significant difference was found. B, C) Intracellular uptake and growth of WT and the recombinant vaccine strains were assessed in J774A.1 macrophage-like cells. The cells were grown as monolayers on coverslips and infected at an MOI of 50:1, and bacterial intracellular growth was measured in the presence of gentamicin at the indicated time points. Data shown are the averages of results from three independent experiments.

3A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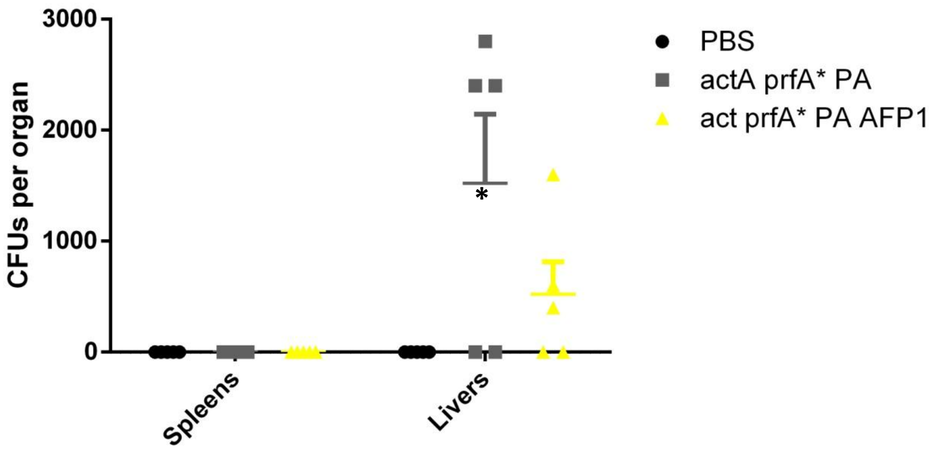
prsA htrA PA AFP2

3B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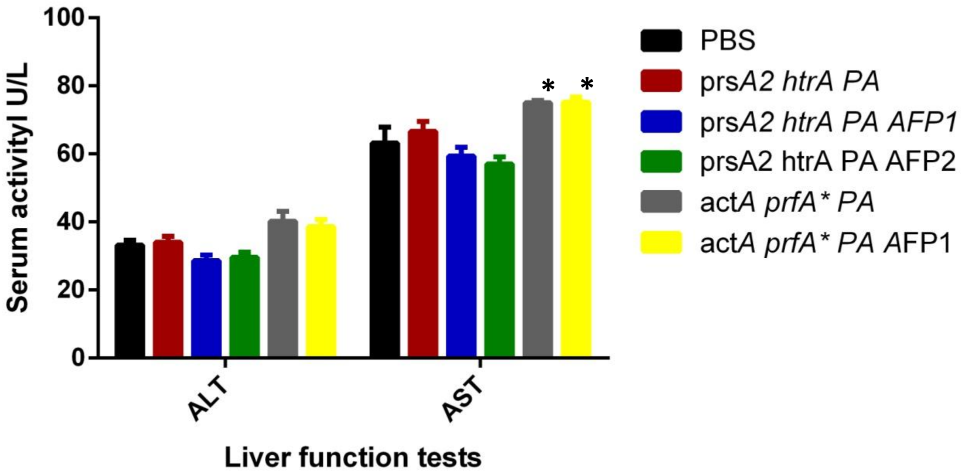
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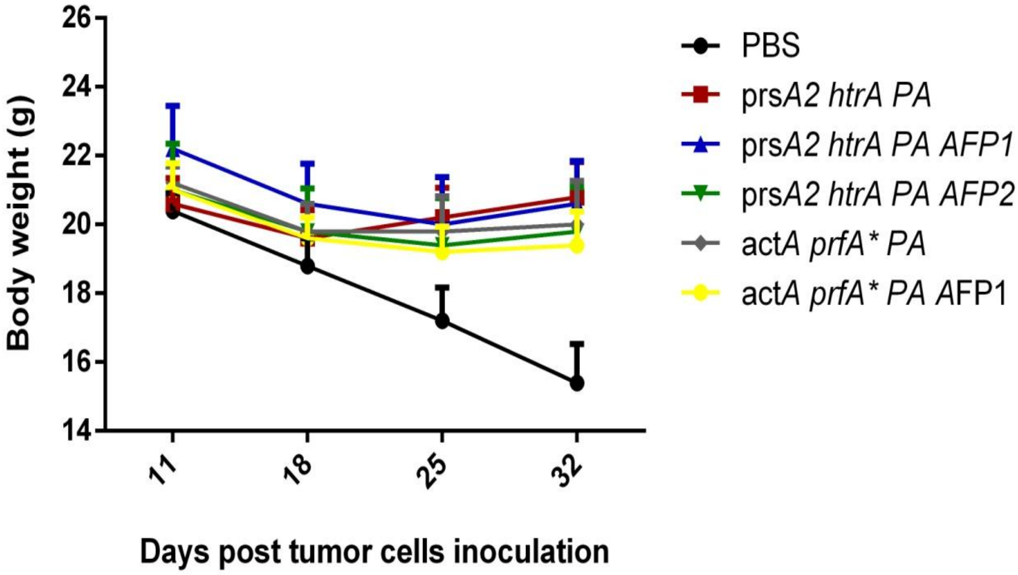

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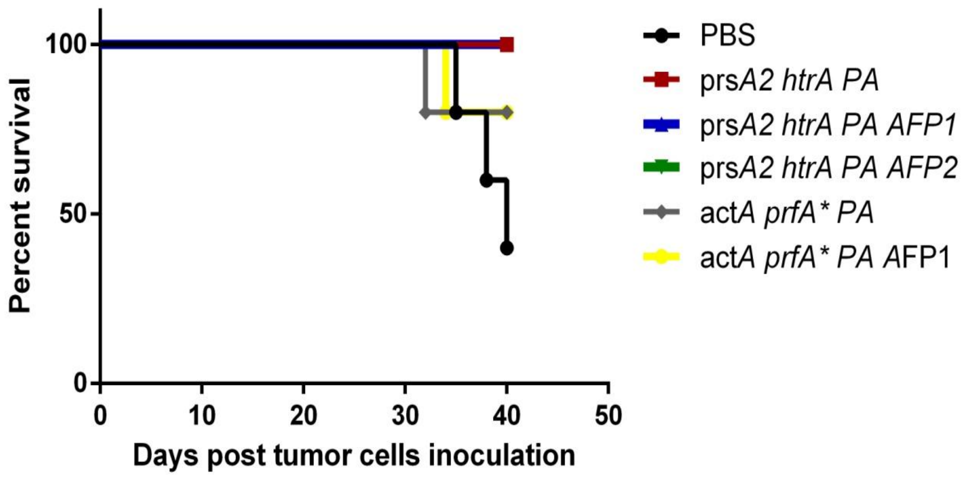
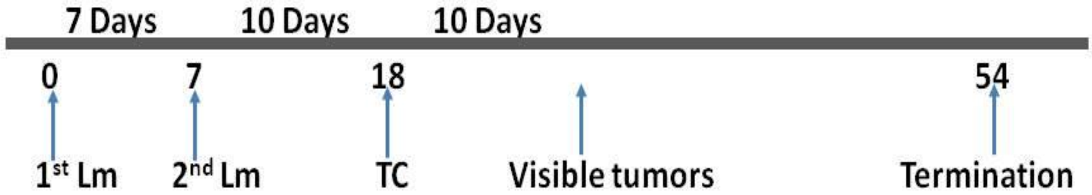


Fig. 3. Determination of safety of *ΔprsA2 ΔhtrA* *L.monocytogenes* recombinant vaccine strains in comparison to ΔactA prfA* strains. A, B) Two immunizations of 10^8 CFUs of ΔprsA2 ΔhtrA strains or 10^7 CFUs of ΔactA prfA*strains were injected intravenously in C57BL/6 mice (n=5). At 72 hours post-last immunization, the livers and spleens were aseptically isolated, homogenized, and plated on BHI plates supplemented with 200µg/ml streptomycin for bacterial CFUs. For A and B, each datum point represents one mouse, and the solid lines denote the median for each data group. C) Blood samples were withdrawn from the retro orbital veins (n=5), and serum was obtained to measure ALT and AST levels. D) The body weights were recorded weekly for one month (n=5). For C and D, statistical significance was determined using two-way analysis of variance with Tukey's multiple comparison test at *P* value of <0.01, and an asterisk (*) sign denotes significance from the WT or PBS group. E) The animals were monitored on daily basis for any death events, and survival proportions were calculated at the end of the study by Kaplan–Meier estimator using GraphPad software version 2.0.

4B). Mice vaccinated with the Δ*actA prfA** strains expressing PA-AFP1 showed enhanced tumor clearance in comparison to mice given PBS, however the initial tumor burdens were significantly larger than those observed for the Δ*prsA2* Δ*htrA* strains (Fig. 4C). Furthermore, mice vaccinated with Δ*prsA2* Δ*htrA* PA-AFP1 strains were the first group to completely clear the tumors (day 22 post-inoculation) while the other groups cleared by day 28 (Δ*actA prfA** PA-AFP1) and day 36 (PBS control) post tumor cell inoculation. Mice vaccinated with strains expressing the PA-AFP2 antigen showed no significant enhancement of tumor regression with the exception of a single time point at day 10 (Fig. 4D). NF-L4365 vaccine has exhibited the greatest protection against HCC; it halted the tumor growth in mice by 80% more than the control group. NF-L4364 and NF-L4366 showed 24% and 63% protection while NF-L4367 and NF-L4368 prevented the tumor progress by 66% and 71% in comparison to the normal group. These data indicate that the highly attenuated *L. monocytogenes* Δ*prsA2* Δ*htrA* strains are not only efficient in stimulating tumor regression but also appear more effective and possibly more attenuated than the previously characterized *L. monocytogenes* Δ*actA prfA** strains.

4A



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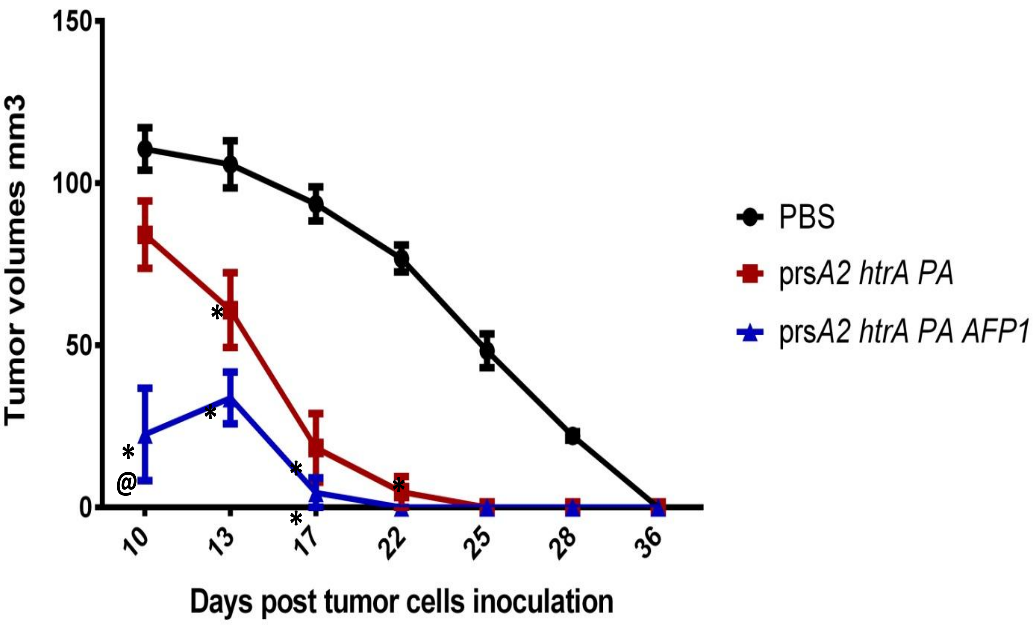

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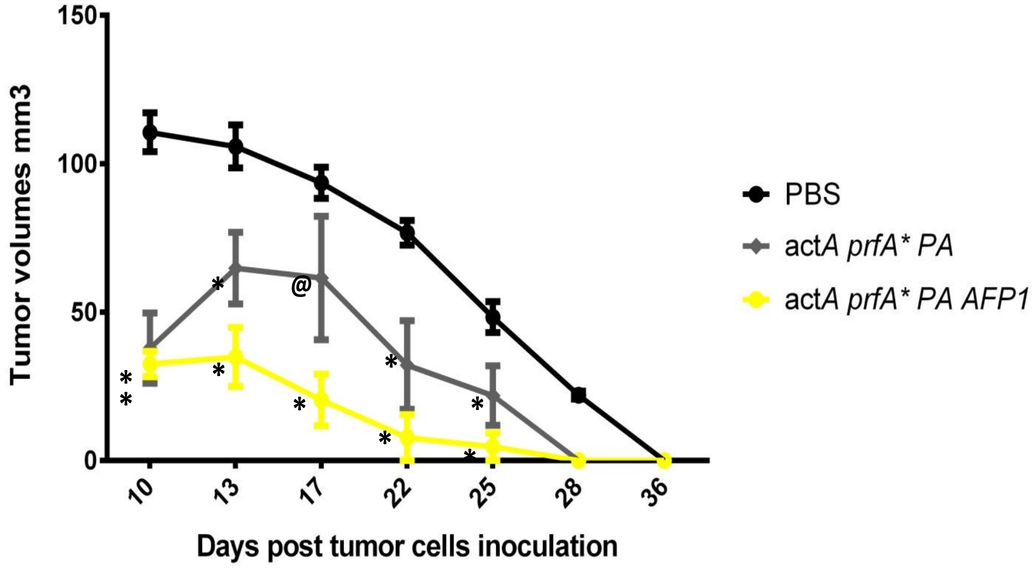

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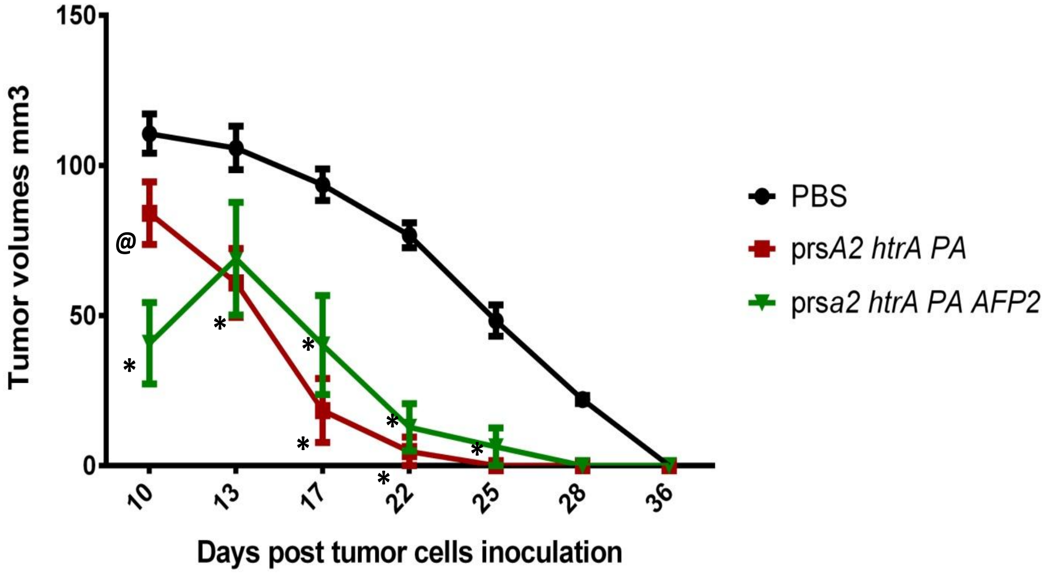


Fig. 4. The efficacy of ΔprsA2 ΔhtrA *L.monocytogenes* recombinant vaccine strains in comparison to ΔactA prfA* recombinant vaccine strains and control against HCC. A) Female C57BL/6 mice 6- 8 weeks (n=5) were immunized with repeated injections of 10^8 CFUs prsA2 htrA and 10^7 CFUs of actA prfA* vaccine strains or 100µl of PBS for the control group as a prophylaxis against HCC tumor cells via retro-orbital injection. Seventeen days later, mice were challenged SC injection of $5x10^6$ Hepa1-6 cells into the flank region; and recording of tumor volumes started when tumors become palpable (10 days after tumor challenge). B, C, D) Tumors volumes were measured every three days by a caliper; the longest side (L) and shortest side (W) were recorded and the volumes (V) were calculated according to the following equation: V= (L X W^2) X 0.5. For B, C and D, n=10 and data shown are presented as mean ± SEM; statistical significance was determined using two-way analysis of variance followed by Tukey's multiple comparison test; a *P* value of <0.01 was considered significant. The asterisk sign (*) denotes significant difference from the control group (PBS) while the (@) sign denotes a significant difference from mice group that received *L.monocytogenes* strains without antigens.

L. monocytogenes ΔprsA2 ΔhtrA strain expressing PA-AFP1 antigen elicits a robust immune response against HCC

To investigate whether a robust immune response was the reason for tumors regression, CD8 T cells were quantified in the splenocytes of control mice and mice immunized with the promising vaccines Δ*prsA2* Δ*htrA* NF-L4364 and NF-L4365 strains. As anticipated, PA-AFP1 fusion generated the highest number of CD8 T cells (Fig. 5A). Although the difference between the vaccines with and without antigens is not significant, it could be confirmed through the difference in tumors and spleens weights between the two groups that Δ*prsA2* Δ*htrA L.monocytogenes* recombinant vaccine expressing AFP1 antigen is activating the immune system more efficiently (Fig. 5B). Hence, *L.monocytogenes ΔprsA2 ΔhtrA* expressing AFP1 antigen represents a safe and effective live attenuated vaccine against HCC cells.

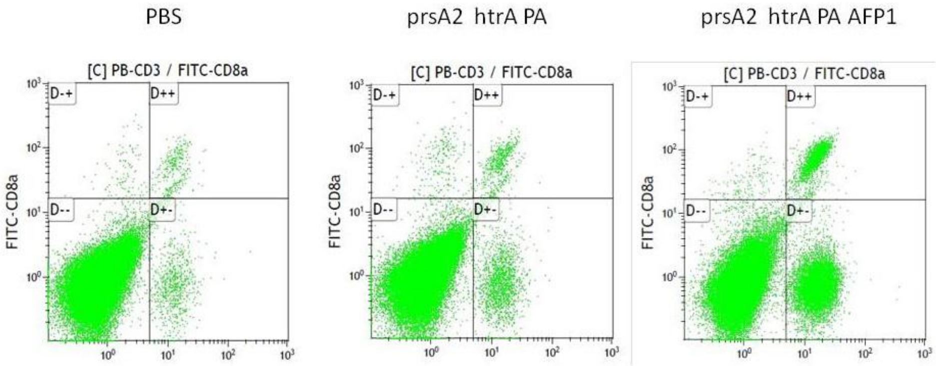
Discussion

Stimulation of effective anti-tumor immune response with little to no patient toxicity is critical for the development of effective immunotherapies for cancer. The elicitation of tumor-specific T cell responses effective against the highly immunosuppressive tumor microenvironment represents a major challenge for successful immunotherapy [29]. Attenuated *L. monocytogenes* strains can be easily configured and adapted to express foreign antigens and generate robust and specific immune responses against cancers [9]. Here we demonstrate that the highly attenuated *L. monocytogenes* Δ*prsA2* Δ*htrA* mutant can be adapted for the safe and effective delivery of tumor antigens, and that vaccination with this modified *L. monocytogenes* protects against HCC progression.

HCC is the most common primary hepatic malignancy; it represents 90% of all liver cancers, and one million new patients are reported annually around the globe [1, 30]. It is a major threat as it remains undiagnosed until end stages [31]. Despite the wide variety of molecular and genetic changes that are found in HCC cells, the tumor is not immunogenic. The tolerogenicity of HCC is maintained due to the normal immunosuppressive environment of the liver; the physiological function of the liver, including removal of pathogens and antigens from blood, makes it an immune-privileged organ [32]. HCC tumors have a dismal prognosis; and in spite of improved radiotherapeutic and chemotherapeutic techniques, fifth year survival is very unlikely. Immunotherapies represent a potential therapeutic option for such tumors, and *L. monocytogenes* appears to be capable of overcoming the immunosuppressive cancer environment [30, 33]. It is a potent delivery vehicle that can induce robust innate and adaptive cellular immune responses specific for recombinant antigens. Importantly, it has been shown that the pre-existence of specific cellular or humoral immunity against *L. monocytogenes* does not reduce vaccine potency [34].

The selection of TAAs is a key component in the development of cancer immunotherapy. Here we have focused on AFP, particularly the immunodominant epitopes $AFP_{137-145}$, $AFP_{158-166}$, $AFP_{325-334}$ and $AFP_{542-550}$, as self antigens for HCC that could be processed and presented to auto reactive T cells in the context of MHC class I [13, 35]. Although it has been argued that AFP-specific T cell responses are difficult to activate, AFP is currently one of the most promising antigens for HCC immunotherapy. Butterfield et al. [36] were the first to reveal that both murine and human T cell repertoires appear to contain self reactive T cell clones that can recognize AFP epitopes, and that AFP-specific T cells were not deleted during the ontogeny of the immune system [37, 38]. Moreover, DNA-based immunizations encoding $AFP_{137-145}$, $AFP_{158-166,}$ AFP325-334, and $AFP_{542-550}$ epitopes have shown protective, but not therapeutic, effect against tumors induced in mice [39]. Although immune tolerance to self-antigens is a significant barrier [40], it appears that the delivery of the selected antigens by highly attenuated *L. monocytogenes* could yield protective and potentially therapeutic effects. PA is secreted with high efficiency by *L. monocytogenes* and it was reasoned that protein fusions to PA might enhance antigen secretion. pPL6-PA-Myc containing PA antigen fusions to *L. monocytogenes* condon-optimized $AFP_{137-145}$ combined with $AFP_{158-166}$ (AFP1), and $AFP_{325-334}$ combined with $AFP_{542-550}$ (AFP2) were generated and introduced in single copy into the *L. monocytogenes ΔprsA2 ΔhtrA* and *ΔactA prfA** chromosomes within a neutral site. The latter have been previously demonstrated to function effectively as vaccine vectors [24].

5A



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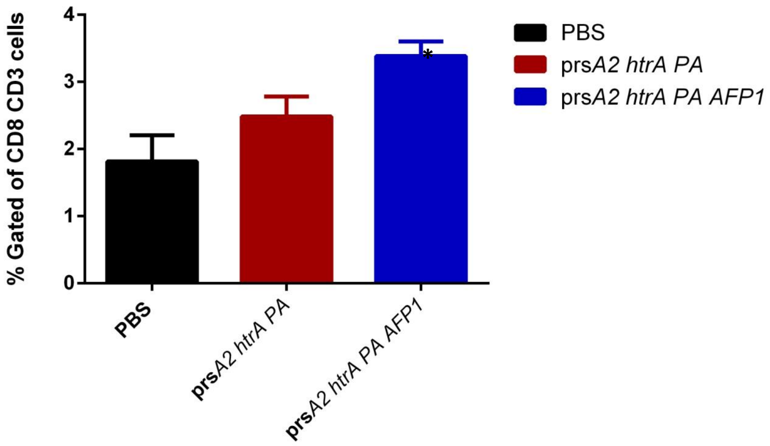

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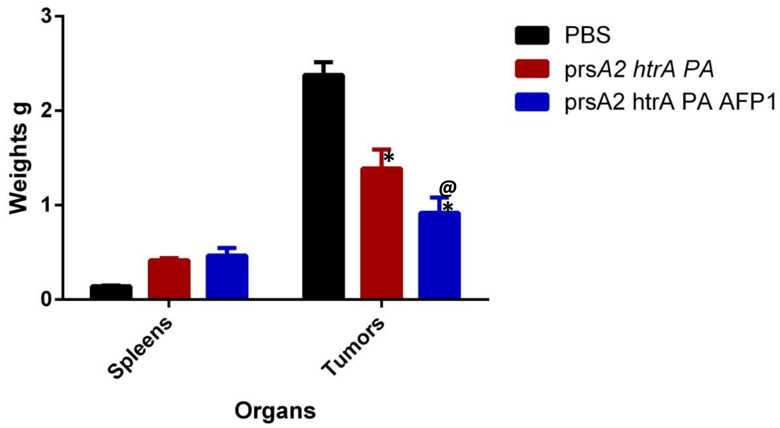

Fig. 5. The effect of *prsA2 htrA* vaccine on eliciting a robust immune response. A) At day 33 of the experiment (15 post tumor inoculation), animals in each group (n=5) were euthanized. Splenocytes were harvested and incubated with the suitable antibodies. After incubation, samples were sent on ice to for detection and quantification of CD8+ CD3+ cells. Kaluza software has been used to extract and analyze the data; and a dot graph has been generated by for each mice. The above dot graphs have been selected as a representative of each group. Quantification of CD8 T cells has been measured as percent gated of CD8+ CD3+ cells in the upper right quadrant of each graph. The bar chart has been done to represent all mice in each group; data are presented as mean ± SEM; and statistical significance was determined using one-way analysis of variance followed by Dunnett multiple comparison test at a P value of <0.05. The asterisk sign (*) denotes significant difference from the control group (PBS). B) At day 33 of the experiment (15 post tumor inoculation), tumors and spleens in each group (n=5) were aseptically isolated, washed with PBS, and their weights were recorded. Data shown are presented as mean ± SEM; statistical significance was determined using two-way analysis of variance followed by Tukey's multiple comparison test; a *P* value of <0.05 was considered significant. The asterisk sign (*) denotes significant difference from the control group (PBS) while the (@) sign denotes a significant difference from *ΔprsA2 ΔhtrA* group with no antigen.

The safety of living vaccine vectors is always a primary concern, yet chosen vectors must still be capable of stimulating host immunity while not themselves presenting a danger for the host. The *L. monocytogenes* double secretion chaperone mutant Δ*prsA2* Δ*htrA* represents a safe, effective delivery vehicle with limited risk associated with infection [41]. Both chaperones have pivotal roles in maintaining survival of *L. monocytogenes* within the host cells. PrsA2 is responsible for folding and stabilization of virulence proteins while HtrA folds but also degrades misfolded proteins to avoid toxic protein accumulation at the membrane cell wall interface. Therefore, in parallel with our data, *L. monocytogenes* Δ*prsA2* Δ*htrA* mutants have previously been shown to be defective for bacterial replication within J774 cells [41]. According to Brockstedt et al [42], the attenuated vaccine strains have a reduced capacity for infecting the nonphagocytic cells, which contributes to its safety. Consistently, our work strongly suggests that the recombinant *L. monocytogenes* Δ*prsA2* Δ*htrA* vaccines are safe with no apparent signs of toxicity following introduction of the bacteria into mice. No mortalities were associated following intravenous injection of *L. monocytogenes* Δ*prsA2* Δ*htrA* and no bacteria were recovered from mouse spleen or liver which are the primary target organs for *L. monocytogenes* replication. In addition, liver function tests based on serum analyses of the immunized mice were similar to those of the control group, and the strains were not able to replicate in the mammalian tissue culture cells. Despite the attenuation of *L.monocytogenes ΔprsA2 ΔhtrA* strain, the recombinant vaccines were able to express and secrete AFP antigens. The fusions cloned into the shuttle integration vector pPL6-PA-Myc were clearly detected by western blot analysis in the cell culture supernatants. Collectively, this gives confidence that *L. monocytogenes* Δ*prsA2* Δ*htrA* constructs would properly function, and likely not result in harmful consequences when used in clinical applications.

Patients with HCC usually suffer from severe cachexia due to remarkable loss in protein mass [43], and weight loss is the direct parameter of measuring cachexia [44]. Therefore mice body weights, in accordance with Hessin et al. [31], were monitored every week. *L. monocytogenes* Δ*prsA2* Δ*htrA* and *ΔactA prfA** vaccines were able to preserve the mice weights; this is probably due to changing of the cytokine profile which was mentioned by Dhanapal and colleagues in 2011 that it could contribute to treatment of cachexia. Weight loss is an important prognostic factor in cancer [44]; thus the retained body weights are not only proving that the vaccines may have no adverse effect on growth, but also lay a foundation of their efficacy. Likewise, monitoring of the survival rate gave evidence about safety as well as efficacy of the vaccines. Different from other investigators that measured the survival rate during 60 days or more [45, 46], we stick to 40 days to comply with the ethics committee recommendations. However, useful insights were gained during this period; *L. monocytogenes* Δ*prsA2* Δ*htrA* vaccines protected the mice and prolonged their survival rate more than the less attenuated vaccine *L. monocytogenes ΔactA prfA** and the control group. Protection against mortality greatly enhances the safety and efficacy profiles of cancer vaccines [47]. To validate the previous statement, more data were generated to prove the extent of effectiveness of *L. monocytogenes* Δ*prsA2* Δ*htrA* vaccine against HCC.

We assessed the efficacy of the vaccines in a prophylactic model of HCC to reflect the clinical situation as much as possible. HCC is clearly a multifactorial event that could be anticipated and rarely happens without underlying chronic liver inflammation [48, 49]. It starts many years after specific risk factors including hepatitis C virus infections, parasitic infections with Schistosoma, aflatoxin intoxication, alpha-1 antitrypsin deficiency and non alcoholic fatty liver disease [50]. In the protection mouse model, *L. monocytogenes* Δ*prsA2* Δ*htrA* vaccine encoding the PA-AFP1 tumor antigens was significantly able to inhibit tumors when compared to non-encoded, AFP2-encoding vaccines and control groups. In accordance, the tumors weights in the PA-AFP1 group were the least. Of particular note, the degree of tumor inhibition by *L. monocytogenes* Δ*prsA2* Δ*htrA* was similar to that of *L. monocytogenes* Δ*actA prfA** vaccine encoding the same antigen. In fact, *L. monocytogenes* Δ*prsA2* Δ*htrA* vaccine cleared tumors entirely from the mice in a shorter period than the *L. monocytogenes* Δ*actA prfA** vaccine. *L. monocytogenes* is of special interest in HCC because it is hepatotrophic and could be designed as a targeted delivery vector to the liver [47]. Attenuated *L. monocytogenes* vaccines retain a comparable degree of immunogenicity to the WT, since the uptake by the antigen presenting cells is not greatly affected by attenuation [42]. Consistently, our data showed that *L.monocytogenes ΔprsA2 ΔhtrA* vaccine encodes PA-AFP1 was efficiently up-taken by mouse macrophages in vitro. And *in vivo*, it stimulated a higher number of CD8 T cells than the *L.monocytogenes ΔprsA2 ΔhtrA* vaccine with no antigen.

Conclusions

We here exploited the typical life cycle of *L. monocytogenes* to construct a relatively safe and effective vaccine. Based on the finding of the current study, it could be concluded that the recombinant strains can readily express and secrete tumor antigens. In addition, the defected in-vitro intracellular growth, and the bacterial burdens recovered from livers and spleens of mice reinforce the assumption that *ΔprsA2 ΔhtrA* strains would be a safer alternative in vivo than the other strains. Another conclusion is that *L. monocytogenes ΔprsA2 ΔhtrA* strain expressing AFP1 has exhibited the greatest protection against HCC; and effectively inhibits the tumors growth in mice. Further investigations would be needed to ensure that a specific immune response was elicited, and transfer the constructs into clinical application.

Conflicts of interest

The authors declare that they have no conflicts of interest.

Acknowledgements

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العلاج المناعي هو طريقة واعدة وفعالة لاستهداف خلايا سرطان الخلايا الكبدية وتحسين نتائج وانقاذ حياة المرضى. لذلك فهناك حاجة ملحة لتطوير اللقاحات الحالية من اجل تحفيذ الجهاز المناعى ضد الخلايا السرطانية فقط وبدون المساس بالخلايا الطبيعية. أثبتت بكتريا Listeria monocytogenes الاختيارية أنها وسيلة فعالة لتصميم لقاحات السرطان التي تحفز استجابات خلايا المناعة CD8 + T . الجوانب الحاسمة لفعاليته تتعلق بقدرة البكتيريا على الوصول إلى سيتوسول الخلايا السرطانية وتقديم مستضدات مرتبطة بالورم. هذا البحث يصف تصميم واختبار سلالات مضعفة من هذه البكتيريا كلقاح فعال ضد سرطان الكبد. تم تصميم Listeria monocytogenes *ΔprsA2 ΔhtrA* للتعبير عن المستضدات البروتينية ألفا المصممة لاستنباط المناعة ضد خلايا سرطان الخلايا الكبدية ، واختبارها في الجرذان جنبا إلى جنب مع سلالة مطورة مسبقًا. تشير النتائج التي توصلنا إليها إلى أن سلالات البكتيريا التى تم تصميمها قد تمثل ناقلاً فعالاً للقاح امن للغاية وفى نفس الوقت قادر على تحفيز المناعة ضد خلايا سرطان الكبد في الجسم الحي.