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Research Article

A Mutant of *Listeria monocytogenes* Shows Decreased Virulence and Confers Protection Against Listeriosis in Mice

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Abstract

This research was performed to obtain a safe and highly immunogenic *Listeria* strain and evaluate the biological characteristics of the deletion mutant. Based on homologous recombination technology, we constructed a deletion mutant *Lm90-ΔinlB* of *L. monocytogenes*. Meanwhile, we characterized its safety and protective efficacy against listeriosis infection in mice. The results showed that the virulence of *Lm90-ΔinlB* could significantly decrease compared with the parental strain *(Lm90)*. The deletion strain retained hemolytic activity and induced CD^{8+} T cell response comparable to that of *Lm90*. Mice immunized with *Lm90-ΔinlB* were capable of stimulating specific CD^{8+} T cells to the listerial epitopes LLO_{91-99} and $P60_{217-225}$ at levels equivalent to *Lm90*. Importantly, immunization of mice with *Lm90-ΔinlB* displayed good protection against listeriosis. In conclusion, strain *Lm90-ΔinlB* is a vaccine candidate with the potential to be more immunogenic yet considerably less toxic than the parental strain.

Keywords: *Listeria monocytogenes, inlB gene, CD^{8+} T cells, Protective efficacy, Vaccine*

Farelerde Düşük Virulans Gösteren ve Listeriosise Karşı Koruma Sağlayan *Listeria monocytogenes* Mutantı

Öz

Bu araştırma güvenli ve yüksek derecede immunojenik bir *Listeria* suşu elde etmek ve delesyon mutantının biyolojik özelliklerini değerlendirmek için yapıldı. Homolog rekombinasyon teknolojisi kullanılarak, *L. monocytogenes*'in bir delesyon mutantı olan *Lm90-ΔinlB* oluşturuldu. Aynı zamanda, farelerde listeriosis enfeksiyonuna karşı güvenliği ve koruyucu etkinliği belirlendi. Sonuçlar, *Lm90-ΔinlB*'in virülansının, parental suşa *(Lm90)* kıyasla önemli ölçüde azalabileceğini gösterdi. Delesyon mutantı suş hemolitik aktiviteyi korudu ve CD^{8+} T hücre tepkisini *Lm90* ile karşılaştırılabilir düzeyde uyardı. *Lm90- ΔinlB* ile immünize aşılanmış farelerde, *Lm90*'a eşdeğer seviyelerde LLO_{91-99} ve $P60_{217-225}$ listeria epitoplarına spesifik CD^{8+} T hücrelerini uyarabilme kapasitesi mevcuttu. Farelerde *Lm90-ΔinlB* ile aşılanması sonrası listeriyoza karşı iyi bir koruma görülmesi önemli bir bulgudur. Sonuç olarak, *Lm90-ΔinlB* suşu, parental suşundan daha fazla immünojenik ancak önemli ölçüde daha az toksik olması nedeniyle potansiyel bir aşı adayıdır.

Anahtar sözcükler: *Listeria monocytogenes, inlB geni, CD^{8+} T hücreleri, Koruyucu etkinlik, Aşı*

INTRODUCTION

Listeria monocytogenes is a Gram-positive pathogen that can cause listeriosis with gastroenteritis, meningitis and encephalitis [1,2]. As a food-borne pathogen, *L. monocytogenes* can cross the intestinal barrier through intestinal epithelial cells or phagocytes and reach the liver and spleen via the lymph and bloodstream. *L. monocytogenes* multiplies rapidly and finally spreads to the brain through blood circulation. *L. monocytogenes* is known to affect pregnant women, immunocompromised individuals, the young, and the elderly via the oral route [3-5]. *L. monocytogenes* has typical characteristics of intracellular parasitism and intercellular transmission, and could simultaneously cause MHC-I and MHC-II antigen delivery system and stimulate the host to produce strong cellular immune response [6,7]. As cytotoxic T lymphocytes (CTLs) are thought to be an important defense against tumor, virus and intracellular bacterial pathogens, attenuated *L. monocytogenes* has the ability to stimulate this immune response, which has broad clinical relevance. At present, several attenuated *L. monocytogenes* have been successfully used in tumor, virus and other DNA vaccine vectors, and some vaccines have entered phase I and phase II clinical trials [8,9].

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L. monocytogenes has abilities to infect not only phagocytes but also non-phagocytes. The infection process can be divided into four stages: invasion, escape phagocytosis, multiplication and transmission between cells. In the process of infection, each step needs to be done by a specific virulence factor. Internalins are the protein products of a family of virulence-associated genes found in pathogenic *Listeria* spp. Internalin A (InlA) and Internalin B (InlB) encoded by the *inlAB* operon were the first members of this family to be characterized. And the two proteins could play important role in *L. monocytogenes* invasion [10]. Specifically, InlA binds to E-cadherin receptor through its LRR region for invading intestinal epithelial cells and trophoblast cells. InlB not only binds to Met receptor through LRR region, but also binds to receptors of gClqR and GAGs through GW region, thus invading hepatocytes, Vero cells and Hela cells [11,12]. Meanwhile, *L. monocytogenes* strongly induces cell-mediated immune responses. As a result of its cytoplasmic location during infection and its particular advantages as a neonatal vaccine vehicle, *L. monocytogenes* can facilitate a long-term cellular immune response, which makes attenuated strains a focus of attention in vaccine development [9,10]. Previous studies have shown that *L. monocytogenes* tropism into hepatocytes is mediated by the virulence factor InlB. Therefore, deletion of *inlB* is expected to limit liver toxicity. Undeniably, *ΔinlB* mutants display reduced hepatocyte entry during the infection of monocytes as efficiently as wild-type strains [11,12].

In this study, we successfully constructed a live-attenuated vaccine strain, *Lm*90-*ΔinlB*. The biological characteristics of the deletion mutant were evaluated at the molecular, cellular and preliminary animal levels, which lays a scientific foundation for the further study of the Listeria vaccine vector and vaccine.

MATERIAL and METHODS

Bacterial Strains and Cell Lines

*Listeria monocytogenes (Lm*90, serotype 4b), isolated from a sheep with encephalitis in Xinjiang, China, was used in this study. An *inlB* deletion mutant (called *Lm*90-*ΔinlB*) was constructed using *Lm*90 as the parental strain. To achieve stationary phase, all the strains were grown in brain-heart infusion (BHI) agar (Oxoid, Basingstoke, UK) or broth without antibiotics at 37°C. For solid media, agar was added at 1.5% (w/v), and chloramphenicol (Cm) from Sigma was added at a concentration of 10 μg/mL.

MBMEC cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37°C with 5% CO_2 for adhesion, invasion and intracellular growth assays. Cell culture media and reagents were all obtained from Gibco.

Animals and Ethical Concerns

BALB/c mice of 6-8 weeks of age were obtained from the Laboratory Animal Research Institute of the China Academy of Medical Sciences. All mice were housed in the facilities with the relatively constant temperature of 25±2°C and were treated in strict accordance with the Ethical Committee for animal use in Shihezi University. During the experiments, every effort was made to minimize animal suffering.

Construction and Identification of Lm90-ΔinlB

A splicing-by-overlap-extension (SOE) technique was utilized to construct the deletion mutant [13]. The sequences of primers *(Table 1)* were designed using Primer 5.0 software (Premier Inc., Canada) according to the sequence within the *L. monocytogenes* F2365 genome in the GenBank database (accession number: AE017262.2). *L. monocytogenes* genomic DNAs were extracted using the DNA extraction kit (Tiangen Bio, Co.). The 5′ upstream and 3′ downstream homologous arms were amplified by polymerase chain reaction (PCR) using primers P1/P2 and P3/P4, respectively. Then mixed the products in a 1:1 ratio and used SOE PCR technique to achieve the fusion fragment with the primer P1/P4. The fusion fragment was recovered and ligated to pMD19-T cloning vector (TaKaRa Bio, Inc.). The *ΔinlB* fragment and shuttle vector pKSV7 were digested with *Eco*RI and *Hin*dIII (Takara Bio, Inc.), respectively. Then the *ΔinlB* fragment was cloned into pKSV7 to obtain pKSV7-*ΔinlB*, and then the recombinant plasmid was transformed into *L. monocytogenes* competent cells by electroporation [14]. Under the pressure

Table 1. *PCR primers used in the study*

Primers	Sequence (5′--3′)	Restriction Site	Product Length (bp)	Target Gene
P1	CCCAAGCTTGGGGGGTCGCTTGTTACTCCA	*Hin*dIII	339	Upstream sequence of the *inlB* gene
P2	GAAATAGCTTTTCGTAGGATAATCCGTACTAAAATC			
P3	TTTTAGTACGGATTATCCTACGAAAAGCTATT		415	Downstream sequence of the *inlB* gene
P4	CGGAATTCCG TACGCAAACTGGCAAAGC	*Eco*RI		
P5	GGGTCGCTTGTTACTCCA		750	Detecting primers
P6	TACGCAAACTGGCAAAGC			
hly-F	CTGAATTCGGCTGTTACTAAAGAGCAGTTGC		743	*hly*
hly-R	ATGGATCCTTAGCCCCAGATGGAGATATTTCTA			

of chloramphenicol and temperature, the recombinant strain was selected on the basis of methods described previously [15].

Growth Curve and Growth Activity Assays of the Bacteria

Bacterial growth was measured by direct optical density detection at 600 nm and enumeration of the colony-forming units (CFU) in serial dilutions plated on BHI agar.

Specifically, after cultured for almost 16 h, appropriate amount of *Lm*90 and *Lm*90-*ΔinlB* was inoculated into fresh medium at the ratio of 1:100 and cultured at 37°C. Bacterial growth was determined every 2 h by the optical density and CFU.

Cell Culture and Infection Experiments

MBMEC cells were cultivated in 24-well plates in DMEM medium containing 10% heat-inactivated FBS at 37°C under a 5% CO_2 atmosphere. For adhesion, invasion and intracellular growth assays, $2x10^5$ cells were seeded in 24-well tissue culture plates 1 to 2 days before infection. Meanwhile, *L. monocytogenes* were cultured to an optical density about 0.3 at 600 nm. Cell layers were washed with PBS and infected in triplicate with 1 mL of bacteria suspended in medium without FBS for 1 h. Then the culture media were collected, diluted and cultured on BHI agar plates to enumerate the bacteria. During the invasion experiment, the medium was replaced with fresh media without FBS and 100 µL of bacterial culture was added into each well. After 1 h of cultivation, the cells were washed and incubated for different time points with medium containing 100 µg/mL^{-1} of gentamicin (Sigma) to kill the extracellular bacteria. Finally, the cells were lysed in 2 mL of DMEM containing 0.2% Triton X-100 (Amresco Inc., USA) to release intracellular bacteria and then the mixture was diluted and spread on BHI agar plates to count the colonies.

Virulence of Listeria Strains In-vivo

Mice were randomly divided into 2 groups and each group had 5 subgroups (n=5). *L. monocytogenes* were cultured overnight, collected by centrifugation and washed with PBS for 3 times. Then the bacteria were diluted by 10-fold gradient in PBS and the mice were intraperitoneally injected with 100 µL of the bacteria with different dilution to determine the median lethal dose (LD_{50}) of listerial strains. The mice were then observed for 14 days and the LD_{50} was measured. The remaining bacteria were cultured on BHI agar plates for bacterial counts. On the 1st, 3rd, 5th and 7th days after intraperitoneal injection of 0.1 LD_{50}, the livers and spleens of mice were sampled quickly to assay the number of bacteria.

Determination of Hemolytic Activity

Hemolytic activity was assayed as described by Portnoy [16] with some modifications. Listerial strains were cultured for 12 h, then the supernatant was collected and the concentration was adjusted according to the OD600 value. Briefly, two-fold serial dilutions of bacterial culture supernatant were made in PBS [pH 6.0] and 70 µL was added per well. After a 30-min incubation at 37°C, 30 µL of 1% sheep red blood cells was added to the sample and mixed. After an additional 30-min incubation at 37°C, the bacterial hemolytic activity was observed and expressed as the reciprocal of the highest dilution. A negative control comprising PBS only was included in the experiment.

Immunization Procedure

Mice were separated into 3 groups of 15 each. On days 1 and 14, one group of mice was immunized intraperitoneally *(i.p.)* with 0.1 LD_{50} of *Lm*90-*ΔinlB* in a total volume of 200 µL. On the same days, mice in the negative control group were given 200 µL PBS, whereas mice immunized *i.p.* with a sublethal dose of *Lm*90 ($2.0x10^4$ CFU per 200 µL) were considered as a positive control group. On day 35, five mice from each group were euthanized for ELISPOT assay and 10 mice were prepared for challenge assay.

ELISPOT Assay

The enzyme-linked immunospot (ELISPOT) assay was used to determine the levels of the major *L. monocytogenes* antigens, LLO_{91-99} and $P60_{217-225}$. To determine the levels of protective immunity of *Lm*90-*ΔinlB*, T cell responsiveness was analyzed using a standard ELISPOT approach, which was performed according to the protocol in the published papers [17-20].

Challenge Assay

According to the immunization procedure above, 10 mice from each group were challenged with 3.0×10^5 CFU per 200 µL of *Lm*90 via the *i.p.* route on day 35. Three days later, all mice were euthanized, then the spleens and livers were collected, homogenized and cultured to determine the CFU of *L. monocytogenes*. Bacteria were enumerated by plating serial dilutions of organ homogenates on BHI agar and incubating 16 h at 37°C.

Statistical Analysis

One-way ANOVA with post hoc analysis by the Dunn's method was used in this study. Differences with a P-value of <0.05 were considered statistically significant. Differences between groups were analyzed using the Statistical Package for Social Sciences software (SPSS 20.0). Graphs were prepared using GraphPad Prism 6.0 graphing software.

RESULTS

Construction of the inlB Deletion Mutant Lm90-ΔinlB

Based on the homologous recombination technology described in the Materials and Methods, we constructed deletion mutant *Lm*90-*ΔinlB*. A single band of 750 bp was amplified from the *Lm*90-*ΔinlB* strain and a good genetic

stability was observed during continuous passage to 20 generations *in vitro (Fig. 1)*, which indicated that *inlB* gene had been deleted from the *Lm*90 genome. With sequencing techniques, we further confirmed that the *inlB* gene had been deleted and had genetic stability *(Fig. 2)*.

InlB did not Affect the Growth of L. monocytogenes

In the study, we measured the growth of *Lm*90 and *Lm*90-*ΔinlB* to examine whether the *inlB* deletion affected the growth of *L. monocytogenes*. The results showed that there were no obvious differences between *Lm*90 and *Lm*90-*ΔinlB (Fig. 3-A)*. In addition, the number of CFU between the two strains also showed no obvious difference *(Fig. 3-B)*. The results in this section indicated that the *inlB* deletion did not affect the growth of *L. monocytogenes*.

Adhesion and Invasion Characteristics of the Deletion Mutant

In the MBMEC cell adhesion assay, *Lm*90 exhibited 4.63% adhesion compared with only 2.52% for *Lm*90-*ΔinlB*. Similarly, in the MBMEC cell invasion assay, *Lm*90 exhibited 0.37% invasion compared with only 0.23% for *Lm*90-*ΔinlB (Fig. 4-A)*. In addition, the number of viable intracellular bacteria for *Lm*90-*ΔinlB* was significantly lower than that of *Lm*90 *(P*<0.05) *(Fig. 4-B)*.

The Virulence of the Deletion Mutant was Reduced in Mice

A notable reduction in virulence was observed with the deletion mutant compared with the parental strain, as shown in *Table 2*. The LD_{50} values for *Lm*90 and *Lm*90-*ΔinlB* in experimental mice were $10^{4.60}$ and $10^{7.38}$ CFU, respectively. The LD_{50} value of *Lm*90-*ΔinlB* was increased by 2.78 orders of magnitude, indicating that *L. monocytogenes* virulence significantly *(P*<0.05) decreased with the lack of *inlB* gene. Furthermore, the number of viable bacteria in the liver and spleen of mice infected with *Lm*90-*ΔinlB* was significantly lower than that of mice infected with *Lm*90 *(P*<0.05) *(Fig. 5)*. In summary, the survival and proliferation of *L. monocytogenes* on days 1-7 post-infection significantly decreased due to the deletion of the *inlB* gene and the virulence of *Lm*90-*ΔinlB* was decreased. Taken together, these results indicated a significant improvement in the safety of *Lm*90-*ΔinlB*.

The Deletion Mutant Retains Hemolytic Activity

The major *L. monocytogenes* antigen listeriolysin O (LLO) confers the unique adjuvant characteristics of *L. monocytogenes* of being able to generate a Th1 immune response. Hemolysis activity results of the *L. monocytogenes* strains were presented in *Fig. 6* and the hemolysis titer of *Lm*90-*ΔinlB* reached 2^5, which was a little lower than that of *Lm*90 (2^6). Therefore, we concluded that *Lm*90-*ΔinlB* had good hemolytic activity.

The Deletion Mutant Induces an Antigen-specific CD8⁺ T Cell Response Similar to the Parental Strain

In the light of immunization procedure in the methods, all experimental mice were vaccinated with primary inoculation

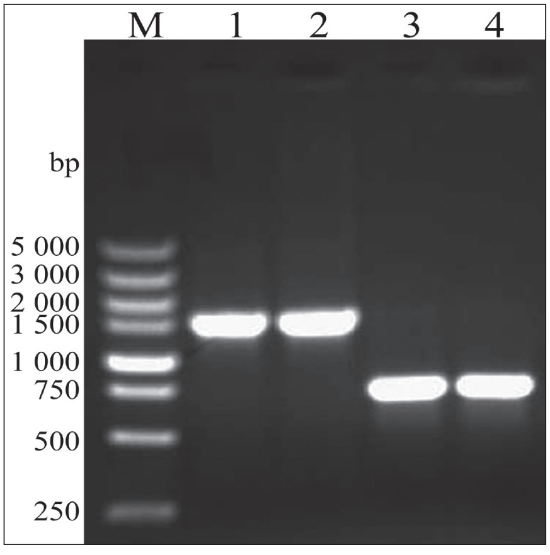

Fig 1. Identification of recombinant strain *Lm*90-*ΔinlB* by polymerase chain reaction. M: DNA marker. 1,2: *Lm*90 used as a positive control. 3: pKSV7-*ΔinlB* used as a positive control. 4: PCR products from *Lm*90-*ΔinlB*

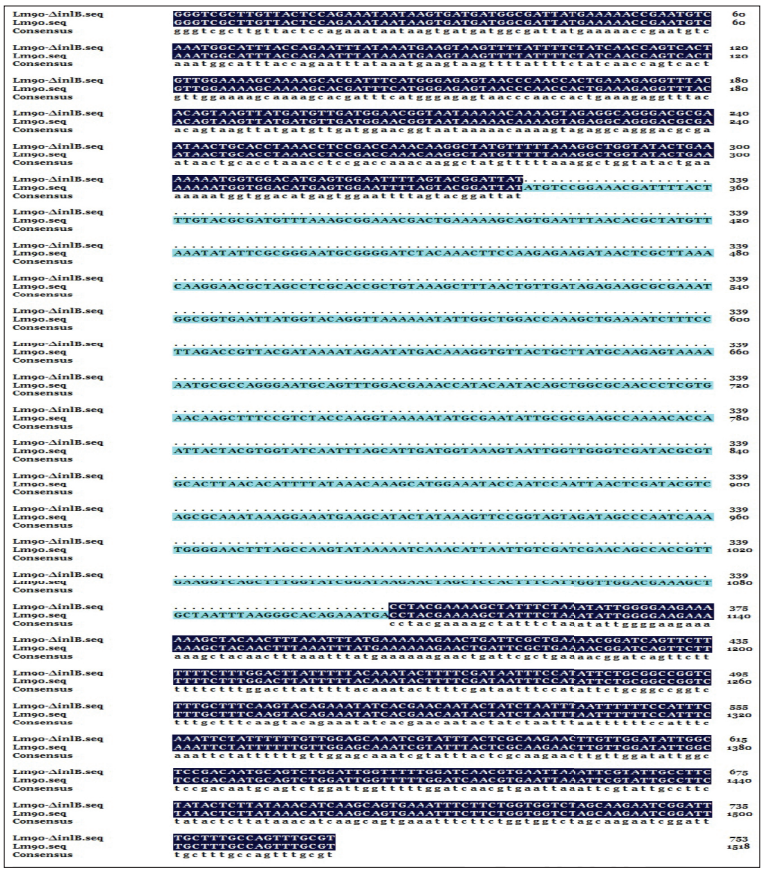

Fig 2. Comparison of sequencing result of amplified products of *Lm*90-*ΔinlB* with the corresponding sequence of *Lm*90

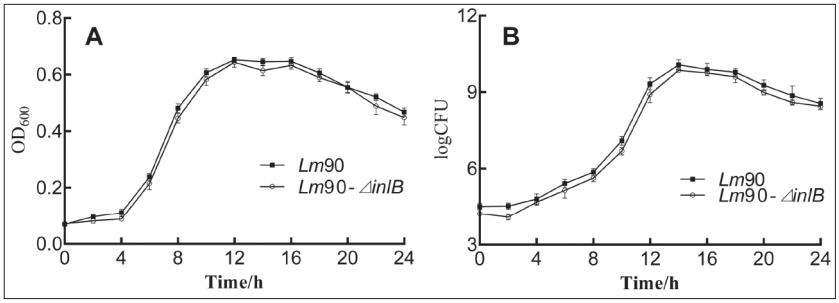

Fig 3. A: Growth curves of *Lm*90 and *Lm*90-*ΔinlB*. Data are from experiments repeated at least three times. B: CFU determination of *Lm*90 and *Lm*90-*ΔinlB*, results are shown as $\log_{10}$ CFU. Bars represent the mean ± SD. No statistically significant differences in growth were observed between strains

Fig 4. A: Results of adhesion and invasion assay in MBMEC cells. * $P<0.05$ according to Kruskal-Wallis one-way ANOVA, with post hoc comparison using Dunn's method. Differences are shown relative to *Lm*90. Error bars represent the mean ± SEM; B: Intracellular growth of *Lm*90 and *Lm*90-*ΔinlB* in MBMEC cells using a gentamicin protection assay. No statistical significance was observed

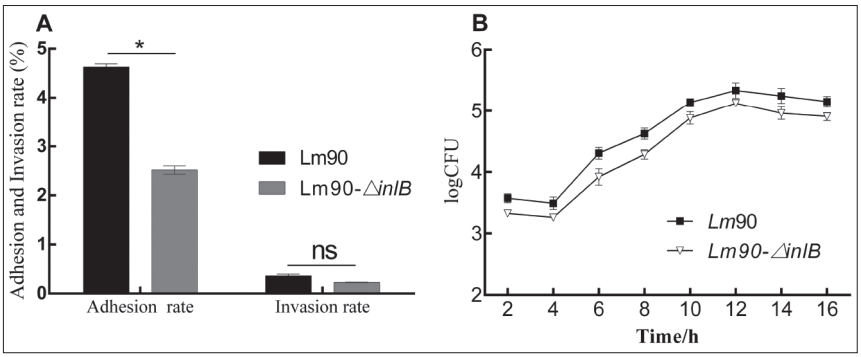

Table 2. *The 50% lethal doses of Lm90 and Lm90-ΔinlB in mice*

Group	*Lm*90			Group	*Lm*90-*ΔinlB*		
	Dose/CFU	Mortality	LD_{50}		Dose /CFU	Mortality	LD_{50}
1	2.0×10^8	5/5	$10^{4.60}$	1	3.0×10^8	5/5	$10^{7.38}$
2	2.0×10^7	5/5		2	3.0×10^7	2/5	
3	2.0×10^6	5/5		3	3.0×10^6	1/5	
4	2.0×10^5	4/5		4	3.0×10^5	0/5	
5	2.0×10^4	2/5		5	3.0×10^4	0/5	

CFU, colony-forming unit; LD_{50}, 50% lethal dose

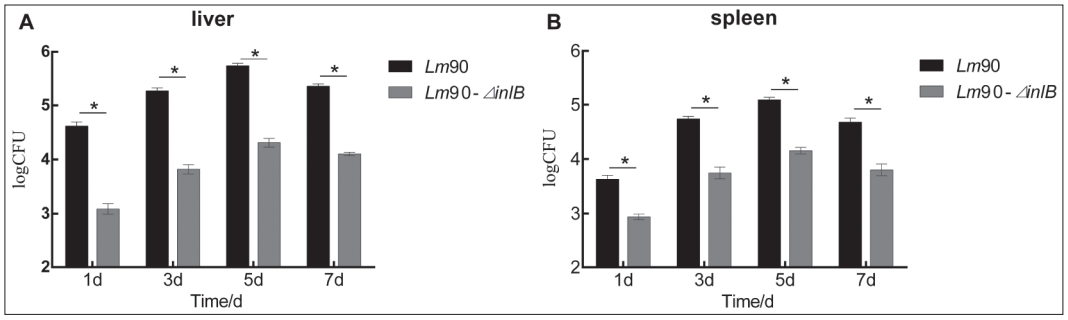

Fig 5. The bacterial count of *Lm*90 and *Lm*90-*ΔinlB* in (A) the liver and (B) the spleen of mice infected by intraperitoneal injection. The data represent three repeats of serial dilutions from one injection. CFU, colony-forming unit. Bars represent the mean ± SEM, and asterisks indicate statistical significance *(P*<0.05)

on day 1 and a booster on day 14. Using a standard ELISPOT method, T-cell responsiveness to $LLO_{91\text{-}99}$ or $P60_{217\text{-}225}$ of mice were examined at day 35 and the results were shown in *Fig. 7*. Mice immunized with *Lm*90-*ΔinlB* elicited $LLO_{91\text{-}99}$-specific $CD8^+$ T cells at levels equivalent to *Lm*90. In the negative control group, no induction of LLO-specific $CD8^+$ cells was observed. Correspondingly, compared with the negative control group, the induction of $P60_{217\text{-}225}$-specific $CD8^+$ T cells showed a statistically significant *(P*<0.01) in the mice immunized with *Lm*90-*ΔinlB* or *Lm*90, respectively.

IP Vaccination with Deletion Mutant (Lm90-ΔinlB) Provides Protection Against Subsequent Parental Strain (Lm90) Challenge in Mice

According to immunization procedure as referred above,

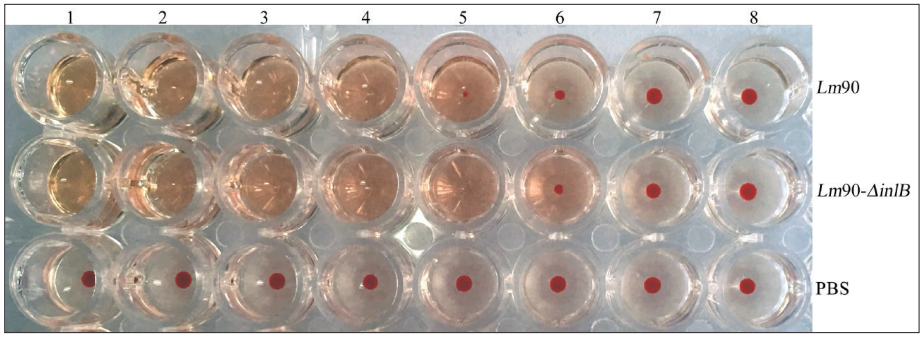

Fig 6. Hemolysis activity test result of mutant strains and parent strain. Culture supernatants were incubated with sheep erythrocytes at 37°C in two-fold serial dilutions (wells 1-8). The mutant strain of *Lm*90-*ΔinlB* had hemolysis titers of 2^5, while the parental strains were 2^6

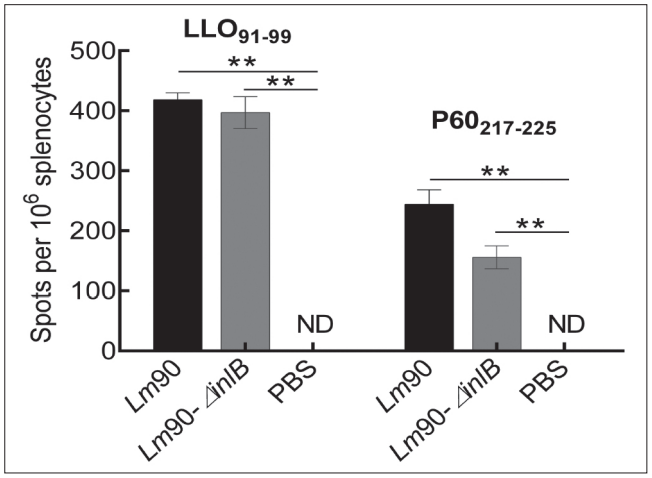

Fig 7. ELISPOT assay. Vaccination injections were given on days 1 and 14 for all murine groups (n=5). Each spot represents one IFN-γ-secreting $CD8^+$ T cell specific to the listerial epitopes LLO_{91-99} and $P60_{217-225}$. Bars represent the mean ± SEM, and asterisks indicate statistical significance (*P*<0.01) compared with the negative control group to which PBS was administered

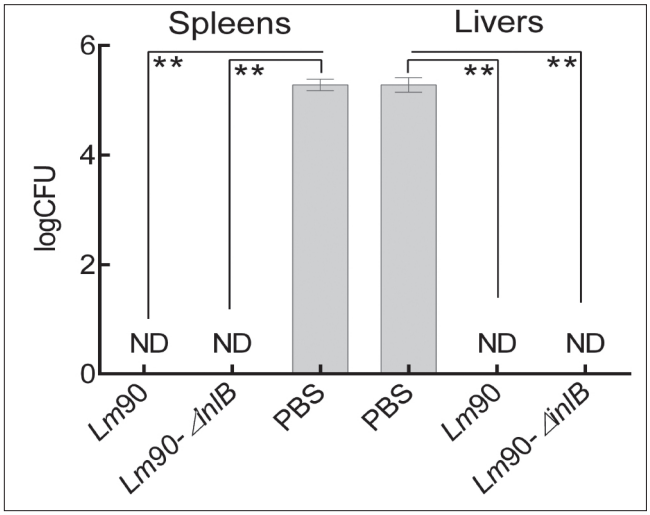

Fig 8. Challenge assay. All murine groups (n=10) were vaccinated via *i.p.* injection on days 1 and 14 prior to challenge with *Lm*90 on day 35. Mice were euthanized 3 days post-challenge and the c.f.u. in the livers and spleens were enumerated. Bars represent the mean ± SEM, and asterisks indicate statistical significance (*P*<0.01) compared with the negative control group to which PBS was administered

the remaining 10 mice from each group were challenged with *Lm*90 on day 35 via the *i.p.* route. Then all mice were euthanized 3 days post-challenge and the spleens and livers were harvested for bacterial counts. By comparison with the PBS-treated group, the mice immunized with *Lm*90-*ΔinlB* showed a protection from listerial challenge and the result was similar to that of *Lm*90 group (*P*<0.01). And the bacterial loads in the organs showed no significant difference between the *Lm*90-*ΔinlB* and *Lm*90 groups (*P*>0.05). On the other hand, the organs of mice in the PBS-treated group had a great deal of bacterial loads after 3 days *(Fig. 8)*.

DISCUSSION

Listeria monocytogenes is capable of provoking strong cell-mediated immune responses and has been widely studied as a model intracellular pathogen. Attenuated listerial strains are often used to convey vaccine antigens since *L. monocytogenes* can induct of major histocompatibility complex (MHC) class I-restricted immune responses [21,22].

In our study, we successfully constructed deletion mutant *Lm*90-*ΔinlB* of *L. monocytogenes*. A product of 750 bp was generated by PCR amplification using recombinant *Lm*90-*ΔinlB* with primers P5/P6. When cultured at 37°C, no significant difference in growth between *Lm*90 and *Lm*90-*ΔinlB* was observed (*P*>0.05, *Fig. 3-A,B)*. It is known that *L. monocytogenes* infects both phagocytes and non-phagocytic cells. The research of Gaillard et al.[23] is valuable to our understanding of the function of internalins. In their study, *L. monocytogenes* was shown to enter into non-phagocytic cells mediated by InlA and InlB. During *L. monocytogenes* infection, InlA promotes listerial uptake into human intestinal epithelial cells (IECs) by means of binding to E-cad [23]. Comparably, InlB has the property to promote invasion into hepatocytes by means of binding to InlB receptor, c-Met. InlB activates c-Met route in the process of invasion and makes easy to enter into cells by inducing actin polymerization [24]. Furthermore, gC1qR and glycosaminoglycans also bind to InlB and promote invasion. Here we focused on InlB and an *inlB* gene deletion strain *(Lm90-ΔinlB)* was constructed in *Lm*90. The virulence of *L. monocytogenes* in mice significantly decreased with the absence of *inlB* and its proliferation in the organs of mice also decreased. In cell adhesion and invasion experiments, the virulence of *L. monocytogenes* significantly decreased with the absence of *inlB* and the

results were in line with previous observation studied by Pentecost et al.[25]. In a murine model, a *ΔinlB* mutant of *L. monocytogenes* produces fewer bacterial counts in the liver [26,27]. Chiba et al.[28] constructed *ΔinlB-Lm* using the 10403s strain and *ΔinlB-Lm* showed less efficiency in invading spleen. In addition, on day 3 after infection, the loads of *ΔinlB-Lm* in various organs were significantly smaller than those of wild-type *L.monocytogenes*. In our study, we also confirmed that the loads of *Lm*90-*ΔinlB* in spleen or liver were significantly smaller than those of the parental strain *(Lm*90). To conclude this section, the results in our study clearly indicated that the importance of *inlB* in the role of regulating virulence and further studies should seek to clarify the involvement of this *L. monocytogenes* virulence factor in pathogenesis.

L. monocytogenes infection induces robust $CD8^+$ T cell responses, which play a critical role in resolving *L. monocytogenes* during primary infection and provide protective immunity against re-infection. It has been shown that both LLO and P60 are major antigen in the protective response against *L. monocytogenes* [29-31]. According to the studies provided by Yamamoto et al.[32] and Kono et al.[33], the secreted protein LLO encoded by the *hly* gene is prominent in generating a Th1 immune response. LLO has been shown to be processed very efficiently into peptides that are presented by MHC class I molecules. LLO_{91-99} is an immunodominant epitope that induces $CD8^+$ CTLs, which protect *in vivo* against *L. monocytogenes* infection and confer significant anti-Listeria immunity on naive mice upon passive transfer [34]. The P60 protein is encoded by the *iap* (for invasion-associated protein) gene [35] and has a notable induction effects on $CD8^+$ T cell response [7]. It is noteworthy that several studies have used live bacterial vectors, such as *Salmonella typhimurium* (*S. typhimurium*) and *Lactococcus lactis*, expressing LLO and/or P60 for vaccination against listeriosis [36,37]. Here we tested the bacterial hemolytic activity of *Lm*90-*ΔinlB* and *Lm*90, and no significant differences were detected between the two strains *(Fig. 6)*. We also showed that mice immunized with *Lm*90-*ΔinlB* could stimulate specific $CD8^+$ T cells against LLO_{91-99} and $P60_{217-225}$ at levels equivalent to *Lm*90. These data indicated that *Lm*90-*ΔinlB* was capable of inducing a powerful *Listeria*-specific T cell response and considerably higher than that of mice inoculated with PBS *(Fig. 7)*.

Previous work, as well as work described by McLaughlin et al.[38], mice immunized with *ΔfrvA* following two *i.p.* immunization doses on days 1 and 14 elicited LLO_{91-99} specific $CD8^+$ T cells at levels comparable with the wild type *L. monocytogenes* on day 35. In our ELISPOT assay, we found that *Lm*90-*ΔinlB* was able to induce $CD8^+$ T cell response effectively, and a challenge assay was carried out to examine whether *Lm*90-*ΔinlB* could be used as a vaccine against listeriosis in mice. The results showed that the mice undergone prime and boost vaccinations, which proved a protective effect against challenge with *Lm*90 *(Fig. 8)*.

In conclusion, our study suggested that *L. monocytogenes* virulence, adhesion, invasion and proliferation were significantly related to the absence of *inlB*. Moreover, *Lm*90-*ΔinlB* retains potent immunogenicity and exhibits significantly decreased virulence compared with parental strain *Lm*90. Our data also demonstrated that the activity of CTLs induced by *Lm*90-*ΔinlB* extremely increased. Importantly, immunization of mice with *Lm*90-*ΔinlB* offered complete protection against listeriosis. This suggests that strain *Lm*90-*ΔinlB* is a vaccine candidate that is more immunogenic yet considerably less toxic than the parental strain and may be used in future work.

Author Contributions

GY and JJ designed the overall study especially the immunization procedure and challenge assay. JR and MY performed all the experiments with assistance and advice from GY, JJ and PW. Laboratory data analysis was performed by MY. The manuscript was written by JR and MY, and reviewed by the co-authors.

Conflicts of Interest

The authors declare that they have no conflicts of interest concerning this work.

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