Recombinant PAL/PilE/FlaA DNA vaccine provides protective immunity against *Legionella pneumophila* in BALB/c mice

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Abstract

Background: *Legionella pneumophila* (*L.pneumophila*), a Gram-negative small microorganism, causes hospital-acquired pneumonia especially in immunocompromised patients. Vaccination may be an effective method for preventing *L.pneumophila* infection. Therefore, it is necessary to develop a better vaccine against this disease. In this study, we developed a recombinant peptidoglycan-associated lipoprotein (PAL)/type IV pilin (PilE)/lagellin (FlaA) DNA vaccine and evaluated its immunogenicity and efficacy to protect against *L.pneumophila* infection.

Results: According to the results, the expression of PAL, PilE, FlaA proteins and PAL/PilE/FlaA fusion protein in 293 cells was confirmed. Immunization with PAL/PilE/FlaA DNA vaccine resulted in highest IgG titer and strongest cytotoxic T-lymphocyte (CTL) response. Furthermore, the histopathological changes in lung tissues of mice challenged with a lethal dose of *L.pneumophila* were alleviated by PAL/PilE/FlaA DNA vaccine immunization. The production of T-helper-1 (Th1) cytokines (IFNγ, TGF-α, and IL-12),

and Th2 cytokines (IL-4 and IL-10) were promoted in PAL/PilE/FlaA DNA vaccine group. Finally, immunized with PAL/PilE/FlaA vaccine raised the survival rate of mice to 100% after challenged with a lethal dose of *L.pneumophila* for 10 consecutive days.

Conclusions: Our study suggests that the newly developed PAL/PilE/FlaA DNA vaccine stimulates strong humoral and cellular immune responses and may be a potential intervention of *L.pneumophila* infection.

Key words: *Legionella pneumophila*; DNA vaccine; PAL; PilE; FlaA

Background

Legionella pneumophila (*L.pneumophila*) is a Gram-negative small microorganism, widely found in nature and man-made water systems[1, 2]. The sporadic, epidemic, or hospital infection of *L.pneumophila* can be fatal, especially in immunocompromised patients[3, 4]. If the patients do not receive timely and correct diagnosis and treatment, the mortality rate of *L.pneumophila* infection can be as high as 50%[5]. Currently, there is no effective measure to prevent *L.pneumophila* infection. Therefore, developing effective, safe vaccine with no toxic side effects to fight *L.pneumophila* infection is of significance.

Early research found that animals artificially infected with *L.pneumophila* could generate a humoral or cell mediated immune response[6, 7]. In addition, compared with the mono-antigen vaccine, the recombinant multi-antigen vaccine has been proven to better induce immunogenicity and stimulate protective immune response [8, 9]. *L.pneumophila* contains multiple virulence factors, such as peptidoglycan-associated lipoprotein (PAL), lagellin (FlaA), and type IV pilin (PilE). PAL is a 19 kDa outer membrane lipoprotein, and as a species distinctive immunodominant component, can be served as a diagnostic indicator for *L.pneumophila* infection[10]. FlaA protein is a key component of *L.pneumophila* flagella. The flagella can enhance the invasion ability of bacteria, which promotes the infection to host cells[11]. Moreover, study suggested that FlaA could play crucial roles in the protective immunity against lethal dose infection of *L.pneumophila* in mice via stimulating T-cell-mediated immune reaction[12]. PilE protein has been demonstrated to facilitate the adhesion between bacteria and their host cells, and is closely related to the DNA transformation of *L.pneumophila*[13]. So far, the effect of recombinant PAL/PilE/FlaA vaccine against *L.pneumophila* infection has not been determined.

Thus, in the present study we selected PAL, PilE, and FlaA for

candidates to construct new recombinant DNA vaccine and investigated its immunogenicity and protective efficacy against *L.pneumophila* in mice.

Results

Construction of recombinant plasmids and expression of recombinant proteins in 293T cells

The full-length gene sequences of PAL, PilE, and FlaA were synthesized and separately cloned into the pcDNA3.1 vector to generate recombinant plasmids of pcPAL, pcPilE, pcFlaA, and pcPAL/PilE/FlaA for expressing PAL, PilE, FlaA, or the fusion protein of PAL/PilE/FlaA, respectively. To verify the expression of these proteins in eukaryotic cells, these recombinant plasmids were transfected into 293 cells. As shown in Fig. 1B, the result of western blotting confirmed the expression of PilE (about 15 kDa), PAL (about 19 kDa), FlaA (about 34 kDa), and PAL/PilE/FlaA (about 70 kDa).

DNA vaccines induced humoral immune in mice

To assess the recombinant DNA vaccines-induced humoral immune response in mice, the IgG titers were detected by ELISA. As presented in Fig. 2, the IgG titers were gradually increased from 1 week to 5 weeks after the enhanced immunization, which were greatly declined at 7 weeks after the enhanced immunization.

Among all these recombinant DNA vaccines, the IgG titer in these groups from high to low, in turn, is PAL/PilE/FlaA, PAL, PilE and FlaA. In the pcDNA3.1 group, the IgG titer was undetectable. So these results suggested that DNA vaccine pcPAL/PilE/FlaA induced a stronger humoral immune response in mice.

DNA vaccines induced CTL response in mice

The spleen lymphocytes were isolated from mice to determine CTL response. As assessed by MTT assay and shown in Fig. 3, compared with pcDNA3.1 group, the CTL response was stronger in PAL, PilE, FlaA, and PAL/PilE/FlaA groups. Among these recombinant DNA vaccine groups, PAL/PilE/FlaA group showed strongest CTL response.

Immunization with recombinant PAL/PilE/FlaA DNA vaccine protected mice against L.pneumophila challenge

To further investigate PAL/PilE/FlaA DNA vaccine-induced protective immunity in mice, the histopathological changes in lung tissues of mice that challenged with a lethal dose of *L.pneumophila* were observed by HE staining. As illustrated in Fig. 4, there was significant inflammatory cell infiltration and destruction of alveolar tissue in the lung tissues of L.pneumophila-infected mice. However, in the lung tissues of mice immunized with PAL/PilE/FlaA DNA vaccine, the inflammatory cell infiltration was obviously restrained.

Moreover, the cytokine response was determined by ELISA. As presented in Fig.5A-C, the serum levels of TNF-α, IFNγ, and IL-10 were significantly up-regulated in PAL/PilE/FlaA group, as compared with control or pcDNA3.1 groups. In the supernatant of splenocyte cultures of mice challenged with a lethal dose of *L.pneumophila*, the levels of TNF-α, IFNγ, IL-12, IL-4 and IL-10 were remarkably enhanced in PAL/PilE/FlaA group after culture for 12, 24, 48, and 72 h (Fig. 6A-E). The survival rate of mice after infected with *L.pneumophila* was monitored for 10 consecutive days. As shown in Fig. 7, the survival rate of mice immunized with PAL/PilE/FlaA DNA vaccine was 100% up to 10 days after infection with *L.pneumophila;* whereas, there were no living mice in control and pcDNA3.1 non-immunized groups from 1 day to 10 days. All the above results indicated that PAL/PilE/FlaA DNA vaccine played an efficient role to prevent *L.pneumophila* infection in mice.

Discussion

In this study, we developed a recombinant PAL/PilE/FlaA DNA vaccine to protect against *L.pneumophila* infection in mice. The results suggested that immunization with recombinant PAL/PilE/FlaA DNA vaccine successfully induced humoral and cellular immunity, alleviated lung inflammation and enhanced the survival rate of *L.pneumophila*-challenged mice.

Recombinant DNA vaccine provides the possibility for production of antigen protein with high purity, which may replace inactivated vaccine and attenuated live vaccine because of its high security and easy production features. The recombinant protein produced by Escherichia coli can not exactly reflect the native structure of bacterial protein, so it is not ideal to evaluate protective efficacy of recombinant protein vaccine in rabbits[14]. After immunization with DNA vaccine, the endogenous antigen protein with natural conformation can be produced by cells within the body, which induces humoral and cellular immunity just like pathogen infection[15]. Compared with recombinant protein vaccine, the titer of produced high affinity antibody is 100-1000 times higher after injection of DNA vaccine[16]. Therefore, compared with traditional vaccines, DNA vaccine has the characteristics of strong and long immune responses, and no virulence reversion. A previous study has indicated that pcDip/pilE DNA vaccine is effective to protect against *L.pneumophila* infection[17]. In our study, we constructed a new DNA vaccine with three protective antigens and evaluated its immune effects.

Gene synthesis is an effective method to obtain DNA template[18]. Gene synthesis is also used to construct DNA vaccine, which avoids the culture of pathogens and lowers the risk for pathogen infection.

Chen et al synthesized the optimized coding sequence of CHA5 to build CHA5 DNA vaccine that induced broad protection against H5N1 influenza viruses[19]. In a recent study, the cDNA sequences encoding full-length Ebola GP and VP40 were synthesized to construct DNA vaccine, which induced specific humoral and cellular immune responses in mice[20]. In this study, the cDNA sequences of PAL, PilE, and FlaA were synthesized and cloned into pcDNA3.1 vector. The expression of PAL, PilE, FlaA proteins and PAL/PilE/FlaA fusion protein were confirmed in 293 cells transfected with recombinant plasmids, which provided a good foundation for the ongoing study.

The ideal vaccine should be an efficient inducer of both humoral and cellular immune responses. To observe the humoral immune responses induced by these recombinant DNA vaccines, we performed ELISA to detect the titer of specific IgG antibody after three times of immunization. According to our results, immunization with PAL, PilE, FlaA and PAL/PilE/FlaA recombinant DNA vaccines could significantly enhance the IgG titer. The results also suggested that PAL/PilE/FlaA recombinant DNA vaccine proved the most obvious effect. The strong CTL response demonstrated that the PAL, PilE, and FlaA proteins played pivotal roles in antigen presentation and subsequent induction of cellular

immune response. Our results indicated that the CTL response was strongest in PAL/PilE/FlaA recombinant DNA vaccine group, compared to that in PAL, PilE, or FlaA group. All these results proved that PAL/PilE/FlaA recombinant DNA vaccine could induce higher humoral and cellular immune responses, so we next evaluated the protective immunity of PAL/PilE/FlaA DNA vaccine against a lethal challenge with *L.pneumophila.*

Helper T cell cytokines are a kind of mediators that have extensively biological activities. T-helper-1 (Th1) cell derived cytokines such as IFNγ, TGF-α, and IL-12 promote the synthesis of IgG2a, and enhance CTL response, which play crucial roles in cellular immune response. While Th2 cell cytokines such as IL-4 and IL-10 contribute to B cell proliferation and IgG1 synthesis, which mainly induce humoral immune response[21]. The balance between Th1 and Th2 cytokines maintains immune homeostasis. The type of immune responses and efficacy of vaccines can be evaluated through the detection of secreted cytokines after vaccination [22, 23]. In the present study, we detected the levels of Th1 cytokines IFNγ, TGF-α, and IL-12, and Th2 cytokines IL-4 and IL-10 at 16 h after *L.pneumophila infection.* We found that the serum levels of TNF-α, IFNγ, IL-10 were increased, and in the supernatant of splenocytes the levels of TNF-α, IFNγ, IL-12, IL-4

and IL-10 were remarkably enhanced after immunization with PAL/PilE/FlaA DNA vaccine. Thus, PAL/PilE/FlaA DNA vaccine elicited both Th1 and Th2 immune responses in mice. Moreover, the survival and histopathological changes in lung tissues of mice were improved by immunization with PAL/PilE/FlaA vaccine. Therefore, protective immunity was induced by PAL/PilE/FlaA DNA vaccine against *L.pneumophila* infection in mice.

There is a lot of debate on the security of DNA vaccine. The primary one is that the vector DNA may integrate into the host genome. However, a lot of research has shown that the integration rate of vector DNA is lower than that of host genome spontaneous mutation [24-26]. In addition, the inoculation methods may affect the safety of DNA vaccine. It has been demonstrated that intramuscular injection and gene gun bombardment are safe inoculation methods for DNA vaccine [27-30]. Based on these studies, the risk of PAL/PilE/FlaA vector integration into host genome was considered to be very low. Although no poisonous side effect was observed in mice after injection of DNA vaccine, the possibility of PAL/PilE/FlaA vector integration into the host genome may exist, which was not assessed in this work. In our subsequent study, we will focus on this issue.

Conclusion

Recombinant PAL/PilE/FlaA DNA vaccine shows higher potential to induce both humoral and cellular immune responses compared with each comprising protein and effectively protects against *L.pneumophila* infection in mice.

Methods

Animals

Six-to-eight-week-old female BALB/c mice (weight about 20 g) were purchased from Liaoning changsheng biotechnology co. Ltd. The mice were housed under a specific pathogen free condition at 22±1 ℃, humidity of 45-55%, and a 12 h light/dark cycle, with free access to food and water.

Bacterial strains, media and growth conditions

Bacteria of *L. pneumophila* serogroup 1 (American Type Culture Collection, USA; no. 35133) were cultured on buffered charcoal-yeast extract agar with buffered charcoal yeast extract (BCYE) (Merck, Germany) in a candle urn at 37 °C with humidified atmosphere and collected with phosphate buffered saline (PBS). After being washed in sterile PBS and centrifuged at 4 °C, the bacteria were diluted to the proper concentration.

DNA vaccine construction and expression in mammalian cells

The cDNA sequences encoding full-length PAL (Gene ID: 19833609), PilE (Gene ID: 19833480), and FlaA (Gene ID:

19832905) antigens were synthesized by Sangon Biotech Co., Ltd (Shanghai, China) and were cloned into the corresponding sites of the eukaryotic expression vector pcDNA3.1 (Invitrogen, Carlsbad, USA). The obtained recombinant plasmids, named as pcPAL, pcPilE, pcFlaA, and pcPAL/PilE/FlaA, respectively, were identified by DNA sequencing. The plasmid profile for pcPAL/PilE/FlaA is shown in Fig. 1A.

293 cells were purchased from Zhong Qiao Xin Zhou Biotechnology Co., Ltd (RZQ0002, Shanghai, China) and were maintained in minimum essential medium (MEM, Gibco, USA) containing 10% fetal bovine serum (Hyclone, USA) at 37 °C under 5.0% CO_2 atmosphere. 293 cells were transfected with the recombinant plasmids pcPAL, pcPilE, pcFlaA, and pcPAL/PilE/FlaA, respectively, by using lipofectamine 2000 (Invitrogen, USA) according to the manufacturer's instructions. After transfection for 72 h, the protein expression was detected by western blotting as described below.

Western blotting

293 cells were lysed in RIPA (Solarbio, China) containing 1 mM PMSF (Solarbio). The protein concentration was evaluated by BCA Protein Assay Kit (Solarbio). Subsequently, 20 μg protein sample was subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride

membranes (Millipore, USA). After being blocked with 5% skimmed milk, the membranes were incubated with Rabbit anti-*L.pneumophila* polyclonal antibody (1:500, MyBioSource, USA) at 4°C overnight. Then the membranes were incubated with Goat Anti-rabbit IgG/HRP antibody (1:3000, Solarbio) at 37°C for 1 h. The bands were visualized by ECL solution (Solarbio).

Immunization of mice

The BALB/c mice were randomly divided into five groups (n=6 per group, total 30), separately immunized by pcDNA3.1, pcPAL, pcPilE, pcFlaA, and pcPAL/PilE/FlaA. Briefly, the mice were intramuscularly injected with 50 μg pcDNA3.1, 50 μg pcPAL, 50 μg pcPilE, 50 μg pcFlaA, and 50 μg pcPAL/PilE/FlaA, respectively. Two weeks and four weeks after the immunization, the mice were injected with the above DNA vaccines at the same dose to enhance immunization. At 1, 3, 5, and 7 weeks after the enhanced immunization, the serum samples were collected and stored at -70℃. The mice were euthanized by an overdose of pentobarbital sodium (200 mg/kg, i.p.), and the spleen lymphocytes were isolated from mice at 7 weeks after the enhanced immunization for further experiments.

Antibody detection

The total immunoglobulin G (IgG) titers were determined by

indirect enzyme-linked immunosorbent assay (ELISA). Briefly, the 96-well plates were coated with 100 μl recombinant PAL/PilE/FlaA antigen at 4°C overnight. After being washed in PBST buffer for three times, the plates were blocked in 5% skimmed milk at 37°C for 2 h. Then 100 μl serial dilutions of serum samples were added to each well and incubation at 37°C for 1 h. Then the plates were washed in PBST buffer for three times and incubated with HRP-labeled Goat Anti-Mouse IgG (1:250, Beyotime, China) at 37°C for 1 h. The plates were then incubated with 200 μl TMB Chromogen Solution (Beyotime) at 37°C for 20 min in the dark. To terminate the reaction, 50 μl of 2M H2SO4 was added to each well. The results were detected at 450 nm by a microplate reader (BioTek, USA).

Measurement of the cytotoxic T-lymphocyte (CTL) response

To assess CTL response, the isolated spleen lymphocytes (5×10^6 /ml, effector cells) from immunized mice were mixed with the cells expressing PAL, PilE, FlaA, and PAL/PilE/FlaA (5×10^5 /ml, target cells), respectively, and then seeded into 96-well plates. The single cultured spleen lymphocytes or PAL, PilE, FlaA, and PAL/PilE/FlaA positive expressing cells were used as effector control or target control. After culture for 56 h, the cells in each group were incubated with 0.5 mg/ml methyl-thiazolyl-tetrazolium (MTT) at

37°C for 4 h. After discarding the supernatant, each well was added with 150 µl DMSO. The absorbance at 570 nm was detected by a microplate reader. The CTL response was evaluated as the following formula: CTL=[1-($A_{570\ effector}$ -$A_{570\ effector\ control}$)]/$A_{570\ target\ control}$×100%.

Studies of protective immunity

The BALB/c mice were randomly divided into three groups (n=16 per group, total 48): control group, pcDNA3.1 group, and pc PAL/PilE/FlaA group, and were intramuscularly injected with equal volume of PBS, 50 µg pcDNA3.1, or 50 µg pcPAL/PilE/FlaA, respectively. The mice were injected with the above DNA vaccines at the same dose to enhance immunization at two weeks and four weeks after the immunization. Two weeks after the enhanced immunization, the mice were intravenously injected with a lethal dose of *L.pneumophila* (2×10^7 CFU in PBS). At 16 h after the injection of *L.pneumophila,* serum samples were collected from 6 mice in each group. Then the mice were euthanized by an overdose of pentobarbital sodium (200 mg/kg, i.p.), and the lung tissues were removed and fixed in 4% paraformaldehyde. The spleen tissues were collected for culture of splenocytes. The remaining 10 mice in each group were monitored for another 10 days for survival analysis.

Hematoxylin-eosin (HE) staining

To observe the pathological changes of the lung tissues, HE staining was performed. The lung tissues were embedded in paraffin and cut into 5-μm sections. Then the sections were subjected to routine HE staining. The results were observed under a light microscope (Olympus, Japan) and the images were taken at a magnification of 200×.

Cytokine response analysis

Cytokine levels in serum samples or the supernatants of splenocytes cultured for 12, 24, 48, and 72 h were detected by commercial ELISA kits for TNF-α, IL-12, IFN γ , IL-4, and IL-10 (USCN Business Co., Ltd, Wuhan, China), according to the manufacturer's instructions.

Statistical analysis

All results were presented as mean ± standard deviation (SD). One-way ANOVA followed by Bonferroni's Multiple Comparison Test was performed to analyze data among different groups using GraphPad Prism 5 software. A P value less than 0.05 was considered to be statistically significant.

Abbreviations

L.pneumophila, *Legionella pneumophila*; PAL,

peptidoglycan-associated lipoprotein; PilE, type IV pilin; FlaA, lagellin; CTL, cytotoxic T-lymphocyte; Th1, T-helper-1; BCYE, buffered charcoal yeast extract; PBS, phosphate buffered saline; MEM, minimum essential medium; IgG, immunoglobulin G; ELISA, enzyme-linked immunosorbent assay; MTT, methyl-thiazolyl-tetrazolium; HE, hematoxylin-eosin; SD, standard deviation.

Declarations

Ethics approval and consent to participate

All animal experiments were conducted in accordance with international ethical guidelines and the National Institutes of Health Guide concerning the Care and Use of Laboratory Animals. The experimental protocol was approved by Shengjing Hospital of China Medical University.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

Funding

This study was supported by grants from the National Key R&D Program of China (No. 2017YFC1309702) and the National Natural Science Foundation of China (No. 81170009). The funders contributed in the study design, data collection and analysis, writing and revising of the manuscript.

Authors' contributions

YYC and YC contributed in the conception and design of the research. YYC, ZHY, and YD contributed to acquisition, analysis, or interpretation of data. YYC wrote the manuscript. YC reviewed and edited the manuscript. All authors read, edited and approved the final version of the manuscript.

Acknowledgments

Not applicable.

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Figure legends

Figure 1 Construction of recombinant expression vector and verification of recombinant protein expression in 293T cells. (A) Construction maps of recombinant pcDNA-FlaA-PilE-PAL. (B) 293T cells were transiently transfected with pcDNA-FlaA, pcDNA-PilE, pcDNA -PAL, pcDNA-FlaA-PilE-PAL or pcDNA 3.1 for 72 h, then the cell lysates were subjected to western blot assay using rabbit *Legionella pneumophila* polyclonal antibody.

Figure 2 Humoral immune responses of the DNA vaccines in immunized mice. 50 μg of DNA vaccines were biweekly intramuscularly injected into the mice for three times. The IgG

titers of multiples groups were detected by ELISA at 1, 3, 5, and 7 weeks after the last immunization.

Figure 3 The cytotoxic T-lymphocyte (CTL) response in mice immunized with the DNA vaccines. At 7 weeks after the last immunization, the spleen lymphocytes were separated. The CTL response was detected by MTT assay.

Figure 4 The histological morphologic changes of lungs in mice challenged with a lethal dose of *Legionella pneumophila*. The lung sections from different groups were stained with hematoxylin and eosin (HE). Scale bar=100 μm.

Figure 5 The cytokine levels of serum samples collected from mice at 16 h after a lethal challenge with *Legionella pneumophila*. The TNF-α (A), IFNγ (B), and IL-10 (C) levels were evaluated by ELISA kits. All data were expressed as mean ± SD (n=6). ***P<0.001 versus the L.p-pcDNA3.1 group.

Figure 6 The production levels of cytokines form splenocytes extracted from mice at 16 h after a lethal challenge with *Legionella pneumophila*. Splenocytes were cultured and their supernatants were collected at 12, 24, 48, and 72 h. The levels of TNF-α (A), IFNγ (B), IL-12 (C), IL-4 (D), and IL-10 (E) were determined by ELISA kits. All data were expressed as mean ± SD (n=3). ***P<0.001 versus the L.p-pcDNA3.1 group.

Figure 7 Protective immunity in mice after a lethal dose challenge with *Legionella pneumophila*. The survival rate of mice for 10 days after a lethal dose challenge with *Legionella pneumophila* was shown (n=10).