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## Protective effect of DNA vaccine with the gene encoding 55kDa antigen fragment against *Pneumocystis carinii* in mice

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### ABSTRACT

**Objective:** To evaluate the protective effect of DNA vaccine with the gene encoding 55kDa antigen fragment of *Pneumocystis carinii* (*P. carinii*) against *P. carinii* in mice. **Methods:** The fragment of the antigen within p55(p55–582) was cloned. Then recombinant plasmid was constructed based on the eukaryotic expression vector pcDNA3.1(+). BALB/c mice were used as experimental models to examine the immunogenicity of pcDNA3.1(+)-p55–582. ELISA and RT-PCR were used to evaluate the role of this kind of DNA vaccine. **Results:** The results of western blot indicated that the recombinant DNA[pcDNA3.1(+)-p55–582] could be expressed correctly and had antigenicity in transfected COS-7 cells. ELISA and RT-PCR showed that pcDNA3.1(+)-p55–582 elicited antibody production, stimulated lymphocyte proliferation and provided partial protection by reducing the *P. carinii* burden. **Conclusions:** The data demonstrate that pcDNA3.1(+)-p55–582 might be potent vaccination that can afford the partial protection for the immunized animals.

## 1. Introduction

*Pneumocystis carinii* (*P. carinii*) pneumonia (PCP), caused by *P. carinii*, remains an important cause of morbidity and mortality in immunocompromised hosts[1]. Although many therapeutic options are used for the treatment of *Pneumocystis* pneumonia, there are adverse side effects, toxicity and resistance in those treatments[2,3]. The development of immunotherapeutic modalities such as active immunization or passive immunotherapy play an increasing important role in the prevention and treatment of infection. Immunization of BALB/c mice with intact *P. carinii* cells provides protection against PCP[4–6]. Since *P. carinii* cannot be satisfactorily cultivated, development of a subunit vaccine is essential. However, there are no convincing data showing that a protective immune response has been elicited after active immunization with an isolated antigen of *P. carinii*[7]. Although DNA vaccination has been used for other *P. carinii* antigen, completely protection against *P. carinii* has not been reported[8].

In our DNA vaccination studies, we focused on the p55 antigen of rat-derived *P. carinii*. It has been reported that purified p55 protein can afford partial protection against the subsequent development of *P. carinii* pneumonia in some animal models. A study has shown that the 5' portion of the molecule, p55 (1–200), appears immunologically silent, failing to elicit lymphocyte proliferation or cytokine secretion[9]. The 3' portion of p55 (268–414), which causes the release of IL-1 and TNF- $\alpha$  in spleen cell suspensions from animals with environmental exposure to *P. carinii*, has at least one immunodominant region. In this study, we tested the efficacy of DNA immunization with p55–582 in eliciting protective immunity of BALB/c mice against *P. carinii*.

## 2. Materials and methods

### 2.1. Amplification and cloning of the target gene fragment

Dexamethasone sodium phosphate (4 mg per week, Jinling, Nanjing, China) was given subcutaneously twice per week to immunosuppress the female Sprague-Dawley rats. Housing and all procedures involving animals were performed according to the guidelines of animal welfare in Nantong University. The total genomic DNA was isolated from homogenized *P. carinii*-infected lung

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tissues of rats and used to perform polymerase chain reaction (PCR). The specific primers (sense primer: 5'-CCGGAATTCACCATGGATTATGTTGT-3' and antisense primer: 5'-CCGCTCGAGTCAATGCGTATTATCTG-3') were synthesized for amplification of a 582 bp DNA fragment of p55 gene. The PCR products were named after p55-582.

This coding sequence was cloned into pGEM-T vector (Promega, Madison, USA). The insert sequence in plasmid was verified by DNA sequencing. The DNA fragment was subcloned into pcDNA3.1(+) at the *EcoR* I and *Xho* I sites for DNA vaccination. To generate recombinant p55/582 protein, the coding sequence was subcloned into the *EcoR* I and *Xho* I sites of a pGEX-4T-1 (Pharmacia, Peapack, NJ). *Escherichia coli* BL21 transformed with the plasmid pGEX-4T-p55-582 was grown at 22 °C for 6 h in the presence of 0.4 mM isopropyl- $\beta$ -D-thiogalactoside (IPTG) prior to purification of glutathione-agarose (Sigma, St. Louis, MO, USA) under denaturing conditions.

## 2.2. Expression of p55-580 protein in COS-7 cells

COS-7 cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum. When 75% confluency was obtained, cells were transfected with 0.1  $\mu$ g of the appropriate plasmid DNA with Lipofectamine (Invitrogen, Shanghai, China) and Opti-MEM reduced-serum medium (Invitrogen, Shanghai, China). The supernatants were separated by SDS-PAGE. The expression of p55-582 in serum was detected by western blotting.

## 2.3. Immunization and challenge

Mice (10 per group) were immunized with 100  $\mu$ g purified pcDNA3.1(+)-p55-582, pcDNA3.1(+) and PBS, respectively. Mice in each vaccination group were immunized four times at 2-week intervals by subcutaneous or peritoneal injection. Animal care and experimental procedures were approved by the Animal Ethics Committee of Nantong University. Blood was collected from mice at day 0, day 14, day 28, day 42, day 56, and allowed to clot. The mice were injected with dexamethasone sodium phosphate, which was given subcutaneously twice per week to immunosuppress the animals at day 56. At day 70, SD rats with ether anesthesia, were challenged with 0.2 mL *P. carinii* suspension through the trachea. Then rats continued injection of dexamethasone in two weeks. All the SD rats were killed at 84 days, and the lung tissues were extracted.

## 2.4. Evaluation

To assess parasite-specific antibody titers, wells were coated with purified antigen GST-p55/582 (1.25  $\mu$ g/mL). Plates were then blocked with 200  $\mu$ L of 1% bovine serum albumin (Sigma, St. Louis, MO, USA) solution overnight at 4 °C. Serum samples were diluted at 1:80 in blocking solution and 100  $\mu$ L were reacted for 1 h at 37 °C. Next, wells were incubated with 100  $\mu$ L of a horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (Sigma, St. Louis, MO, USA) for 1 h at 37 °C in a moist chamber. The plate was developed with 100  $\mu$ L of peroxidase substrate for 5 minutes. The plates were read by ELISA microtiter plate reader at 490 Å and optical density (OD) was determined.

Single cell suspensions were prepared at day 56 (two weeks after the fourth immunization). Splenocytes were co-cultured with ConA (10  $\mu$ g/mL, sigma) in RPMI-1640 for 58 hours before addition of MTT (10  $\mu$ L per well), and incubated at 37 °C for 4 h. For each pellet, 100  $\mu$ L of dimethylsulfoxide

(DMSO) was added to dissolve the formazan for 10 minutes. Absorbance was measured by ELISA plate reader with a test wavelength of 570 nm and a reference wavelength of 630 nm to obtain sample signals.

## 2.5. RT-PCR analysis

The *P. carinii* burden was determined by real-time PCR quantification of the single-copy DHFR gene as described previously<sup>[10]</sup>. The specific primers (5'-TAGATCCAGAGATTCATTTTCGAG-3') and (5'-GTTGCACTTACAACCTTCTTATGG-3') were designed to amplify a 234-bp fragment of the single-copy DHFR gene (AF322061). Duplicate reactions were performed using SYBR Premix Ex Taq™ (TaKaRa Biotechnology, Dalian, China) with 0.2  $\mu$ M of each primer and 2  $\mu$ L of cDNA. Primers for  $\beta$ -Actin (5'-CCCATCTATGAGGGTTACGC-3', 5'-TTTAATGACACGCACGATTTTC-3') were designed using Primer5.0 Software. Cycling conditions were: 15 s at 93 °C followed by 40 cycles of 95 °C for 5 s, 60 °C for 15 s and 72 °C for 20 s. Melt curve analysis and 2% agarose gel electrophoresis of the amplicon were used to determine specificity of the amplicon. Data was normalized with  $\beta$ -actin and results were expressed as fold amplification compared with normal and uninfected mice.

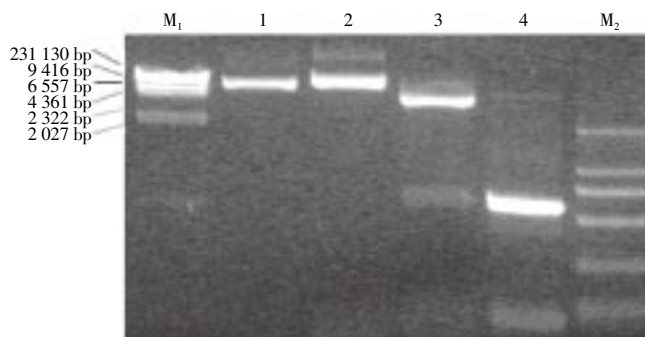
## 2.6. Statistical analysis

Data are presented as the means  $\pm$  standard deviations and were considered significant at  $P < 0.05$ . Data were evaluated for statistical significance by one-way analysis of variance.

## 3. Results

### 3.1. Construction of pcDNA3.1(+)-p55-582

To evaluate immunogenicity of p55/582 protein, we amplified a 582-bp DNA fragment from *P. carinii* genomic DNA<sup>[11]</sup> and constructed a recombinant plasmid named pcDNA3.1(+)-p55-582. The insert fragment was confirmed by restriction enzyme digestion and DNA sequence analysis. Figure 1 showed that the length of the available products by the double-digested with *EcoR* I and *Xho* I endonucleases was identical to the length of the PCR products. All the data reveals that the recombinant eukaryotic plasmid pcDNA3.1(+)-p55-582 has been constructed successfully.

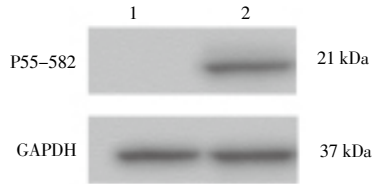


**Figure 1.** Characterization of the recombinant plasmid pcDNA3.1(+)-p55-582 by restriction enzyme digestion.

M1: Marker  $\lambda$ -Hind III digest; M2: Marker DL2000; Lane 1: pcDNA3.1(+)-p55-582 plasmid; Lane 2: pcDNA3.1(+) plasmid; Lane 3: pcDNA3.1(+)-p55-582 plasmid digested with *EcoR* I and *Xho* I; Lane 4: 582 bp DNA fragment of p55 gene.

### 3.2. Expression of p55–582 in COS–7

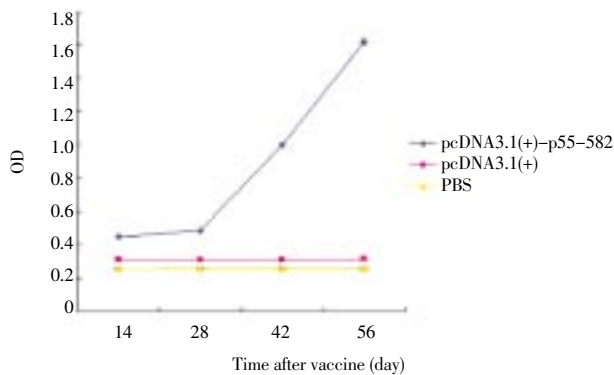
The ability of the recombinant DNA plasmids to express their antigens was evaluated in COS–7 transfected cells. Cells transfected with pcDNA3.1(+)-p55–582 showed positive, while cells transfected with pcDNA3.1(+) were negative. As shown in Figure 2, a band with a molecular weight of 21 kDa, which corresponds to the expected molecular weight of the p55–582 protein, was detected in cell lysates by western blotting.



**Figure 2.** Analysis of p55–582 protein expression by western blotting. COS–7 cells were transfected with pcDNA3.1 (Lane 1) and pcDNA3.1(+)-p55–582 (lane 2). Anti-GAPDH was used as a control.

### 3.3. Strong immune response of BALB/c mice immunized with pcDNA3.1(+)-p55–582

The ability of immunization to elicit humoral and cell mediated immune responses to pcDNA3.1(+)-p55–582 were evaluated by ELISA and lymphocyte proliferation assay. Serum antibodies against by ELISA showed a significant increase over control animals immunized with pcDNA3.1(+) or PBS alone at all time points (Figure 3). Antibody levels were at peak after the fourth immunization in animals. Antibodies to p55–582 were detected in animals immunized with pcDNA3.1(+)-p55–582 at two week after the first immunization (day 14) and these persisted throughout the period of immunization. Antibody levels were significantly higher than antibodies detected in animals immunized with pcDNA3.1(+) ( $P < 0.05$ ). Spleens were isolated to determine cellular immune responses to p55–582 protein. The proliferation responses to p55–582 protein were significantly greater in splenocytes from pcDNA3.1(+)-p55–582 immunized mice than in those from control mice ( $P < 0.05$ ) (data not shown).

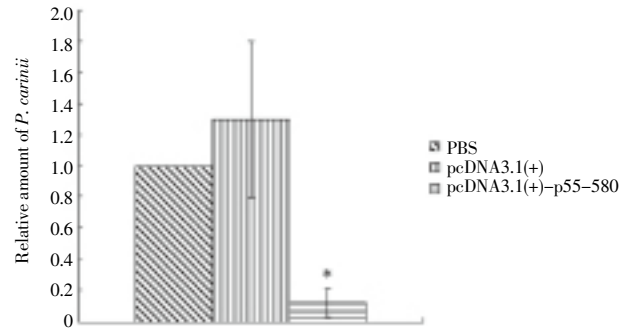


**Figure 3.** ELISA analysis of antibody responses to pcDNA3.1(+)-p55–582 antigen. Significant differences ( $P < 0.05$ ) existed from values for control group [pcDNA3.1(+) and PBS group] on day 14, day 28, day 42, day 56, respectively, as calculated by the means – standard deviations.

### 3.4. Challenge of immunized mice

pcDNA3.1(+)-p55–582 vaccine candidates were evaluated

for their ability to induce protective immunity against lethal challenge of *P. carinii* by real-time PCR quantification of the single-copy DHFR gene. As shown in Figure 4, reduction in organism burden was greater in animals immunized with pcDNA3.1(+)-p55–582 ( $0.124 \pm 0.092$ ) versus the animals immunized with pcDNA3.1(+) ( $1.297 \pm 0.500$ ) ( $P < 0.05$ ). It showed that immunization with p55–582 protein may induce protection against *P. carinii* challenge, compared to that observed in pcDNA3.1(+) inoculated mice.



**Figure 4.** The *P. carinii* burden measured by RT-PCR.

Significant differences ( $P < 0.05$ ) existed from values for control group [pcDNA3.1(+) and PBS group] on day 14, day 28, day 42, day 56, respectively, as calculated by the means – standard deviations.

## 4. Discussion

*Pneumocystis* is a common fungal opportunistic pulmonary pathogen of compromised hosts. Although many therapeutic options are available for the therapy of *Pneumocystis* pneumonia, the treatment is often complicated by side effects, toxicity, and markers of drug resistance [2,3]. Although previous studies have examined the host immune response to *P. carinii*, most have examined the response to specific *P. carinii* antigens because of the difficulty *in vitro* culture of *P. carinii*. Earlier studies characterizing the immune response to MSG demonstrated that it does elicit a T-cell response in rats and SCID mice [12,13]. However, evidence shows that MSG belongs to a family of genes and one or numerous species of MSG may be represented [14–17] by purifying native antigen. In our previous study, we cloned the full-length *Pneumocystis kexin* gene and found this protein can induce immune defense against *P. carinii* infection [18]. The availability of a recombinant antigen has permitted studies focusing on the cellular and humoral responses to a single antigen of *P. carinii*, p55. Several laboratories have shown that p55 is capable of eliciting cellular and humoral immune responses in animals naturally infected with *P. carinii* [11,19]. Compared with the whole extracts of *P. carinii* or the major surface antigen, MSG, the immunization with the entire p55 molecule is necessary in protection against subsequent *P. carinii* infection. Hence, this antigen moiety will likely be most useful as a vaccine candidate in combination with other immunogens which provide similar partial protection.

Several studies about the immune response to p55 have shown that similar or improved levels of protection were afforded by an carboxyl terminus fragment of p55 containing amino acid residues 268–414, which contains a 10 tandem repeats of a glutamic acid-rich 7-amino acid motif, compared to either the full molecule or a amino terminal fragment containing residues 1–200 [29]. A synthetic peptide containing two copies of this repeat did stimulate a specific T-cell response, indicating that this region is a T-cell epitope present in the protein [2].

Although *P. carinii* pneumonia occurs predominantly

in situations of T-cell dysfunction in humans and animal models, several studies have demonstrated the importance of antibodies in the host response to *P. carinii*. Antibodies do not appear to have a direct lethal effect on *P. carinii*, but may be likely to function as enhancers of macrophage phagocytosis[20,21]. In the model system used in the present study, antibodies was detected after 2 weeks of immunization with the pcDNA3.1(+)-p55-582. Significantly higher antibody titers against p55-582 were demonstrated among animals immunized with pcDNA3.1(+)-p55-582 compared with control animals. We have further expanded these studies to the immunogenicity of the p55-582 by western blot. The recombinant protein, p55-582, can be well recognized by the animal immunized with pcDNA3.1(+)-p55-582, while it can not response to the serum from the animal immunized with pcDNA3.1(+), indicating that p55-582 protein has at least one immunodominant region. In the present study, we have demonstrated a lymphocyte proliferative *in vitro* from BALB/c mice immunized with the pcDNA3.1(+)-p55-582. Compared to control animals immunized with the pcDNA3.1(+)-p55-582 did elicit a vigorous proliferative response in splenocytes from the immunized animals ( $P < 0.05$ ). It reveals that the p55-582 protein is capable of eliciting cellular immune responses in immunized animals.

The efficacy of this model in this study has largely been assessed based on reduction of organism burden by real-time PCR other than lower lung weight to body weight ratio. Studies have shown that molecular methods for the detection of *P. carinii* can offer objectivity as well as the potential for increased sensitivity and specificity compared with lower lung weight to body weight ratio[22]. One of the main advantages of real-time PCR is that amplification and detection are performed in a closed system, reducing the possibility of run-to-run contamination. In addition, real-time PCR assays have rapid turnaround times and present a level of objectivity that the current staining methods do not offer. In our study, organism burden by real-time PCR showed greatest reduction in pcDNA3.1(+)-p55-582 immunized animals ( $P < 0.05$  compared to controls), indicating that p55-582 protein can offer partial protection against *P. carinii*.

A detailed understanding of the host-parasite interaction will facilitate the development of immunoprophylaxis and immunotherapy for *P. carinii* infection. pcDNA3.1(+)-p55-582, as a candidate DNA vaccine, can lead to strong cellular immunity, humoral immunity and reduce the organism burden in mice. Additional studies are needed to identify the mechanism of protection and optimize the level of protection afforded by p55-582 protein in the loss of CD4<sup>+</sup> T cells predisposes mice.

### Conflict of interest statement

We declare that we have no conflict of interest.

### Acknowledgments

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