Vaccination with Recombinant Non-transmembrane Domain of Protein Mannosyltransferase 4 Improves Survival during Murine Disseminated Candidiasis

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Candida albicans is the most common cause of invasive fungal infections in humans. The C. albicans cell wall proteins play an important role in crucial host-fungus interactions and might be ideal vaccine targets to induce protective immune response in host. Meanwhile, protein that is specific to C. albicans is also an ideal target of vaccine. In this study, 11 proteins involving cell wall biosynthesis, yeast-to-hypha formation, or specific to C. albicans were chosen and were successfully cloned, purified and verified. The immune protection of vaccination with each recombinant protein respectively in preventing systemic candidiasis in BALB/c mice was assessed. The injection of rPmt4p vaccination significantly increased survival rate, decreased fungal burdens in the heart, liver, brain, and kidneys, and increased serum levels of both immunoglobulin G (IgG) and IgM against rPmt4p in the immunized mice. Histopathological assessment demonstrated that rPmt4p vaccination protected the tissue structure, and decreased the infiltration of inflammatory cells. Passive transfer of the rPmt4p immunized serum increased survival rate against murine systemic candidiasis and significantly reduced organ fungal burden. The immune serum enhanced mouse neutrophil killing activity by directly neutralizing rPmt4p effects in vitro. Levels of interleukin (IL)-4, IL-10, IL-12p70, IL-17A and tumor necrosis factor (TNF)-a in serum were higher in the immunized mice compared to those in the adjuvant control group. In conclusion, our results suggested that rPmt4p vaccination may be considered as a potential vaccine candidate against systemic candidiasis.

Key words Candida albicans; systemic candidiasis; recombinant protein vaccine; Pmt4p

Candida albicans is a human commensal and its interaction with the host immune system plays an important role in both commensalism and infection. The systemic candidiasis occurs predominantly as a consequence of some high-risk medical procedures, immunosuppressive therapy, and aging. It affects organs such as the brain, liver, spleen, lungs, eyes, heart, and kidneys, leading to abscess formation and organ failure associated with mortality in approximate 50% of all cases, irrespective of the administration of intensive antifungal therapy. Because of the rising incidence of life-threatening candidiasis and high failure rates of treatment, it is highly desirable to identify new vaccine targets for prevention and intervention. Different antigenic targets of C. albicans have been investigated with the ultimate aim of generating immunological tools to combat Candida infections. To date, fungal cell-wall polysaccharide, proteins, DNA and live attenuated fungi have been investigated as vaccination targets. These candidate vaccines may be useful in preventing systemic candidiasis in some categories of at-risk subjects before they become heavily immunocompromised. Humoral immunity provides substantial protection against C. albicans growth. Cellular immunity against systemic candidiasis is important as well. Both CD4+ and CD8+ T cells are required in immunized animals to induce the resistance to systemic candidiasis; passive transfer of sensitized T cells can promote removal of the fungi.

Fungal cell walls are essential organelles and are composed of molecules that are largely absent in mammals. The cell wall plays important role in cell wall biosynthesis, growth, budding, and virulence in C. albicans. Most virulence factors, including adhesion, invasion, and yeast-to-hypha transition, localize on the cell wall, such as Pga4p, Pga7p, Alg2p, Pmt4p, Mnn46p, Iff11p. The cell wall also plays an important role in crucial host-fungus interactions that facilitate the establishment of human mycoses. Thus the cell wall proteins might be ideal vaccine targets to induce protective immune response in host. Meanwhile, protein that is specific to C. albicans is also an ideal target of vaccine. In this study, 11 proteins involving cell wall biosynthesis, yeast-to-hypha formation, or specific to C. albicans were chosen and were successfully cloned, purified and characterized. Further protective immune responses of these 11 recombinant proteins were investigated in BALB/c mice, respectively. Among them, protein mannosyltransferase 4 (Pmt4p) belongs to the PMT gene family in C. albicans which encodes five isoforms of protein mannosyltransferases initiating O-mannosylation of secretory proteins. Pmt4p locates on the cell wall of C. albicans and is required for cell wall composition, hyphal growth, and virulence. The recombinant rPmt4p vaccination resulted in significant improvement in survival rate. The induced protective humoral and cellular responses upon rPmt4p vaccination were further investigated.

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MATERIALS AND METHODS

Mice  Male and female BALB/c mice, ranging in age from 6 to 8 weeks, with a weight of around 18–22 g, were obtained from SLAC Laboratory Animal Co., Ltd., Shanghai, China. All animal studies were carried out at the Animal Facilities of the School of Pharmacy at Second Military Medical University, China. All mice were housed in controlled temperature (23 to 25°C) and lighting (8:00 a.m. to 8:00 p.m. light, 8:00 p.m. to 8:00 a.m. dark) and with free access to standard food and drinking water. All animal experiments were approved by the Institutional Animal Care and Use Committee of Second Military Medical University, and conformed to the National Institute of Health guidelines on the ethical use of animals. The number of animals used in the experimentation was minimized for ethical reasons.

Strains and Plasmid  C. albicans SC5314, a well-characterized clinical isolate that is highly virulent in mice models, was kindly provided by Dr. William A. Fonzi (Georgetown University, Washington, D.C., U.S.A.), and was cultured at 30°C in YPD (1% yeast extract, 2% bacto peptone, 2% glucose) medium. Plasmid pYES2/CT (Invitrogen, U.S.A.) was designed for inducible expression of recombinant proteins in Saccharomyces cerevisiae. The pHYES2/CT (5936 bp) contains GAL1 promoter, C-terminal Simian virus 5 (V5) epitope and His tag is approximate 5 kDa. Plasmids were maintained in Escherichia coli DH5-alpha.


The target fragment of each gene was then cloned into the pHYES2/CT vector to generate plasmid pHYES2/CT-target-fragment. The right-oriented plasmid pHYES2/CT-target-fragment was linearized by digestion with corresponding restriction enzymes and transformed into S. cerevisiae INVScl.

The expression of target recombinant proteins was induced when the yeast cells containing pHYES2/CT-target-fragment were grown for 12 h at 30°C in SC medium supplemented with galactose. Induced yeast cells were collected by centrifugation, washed twice with distilled water, resuspended in breaking buffer (50 mM sodium phosphate, pH 7.4; 5% glycerol; 1 mM phenylmethanesulfonyl fluoride (PMSF)), and lysed mechanically with an equal volume of glass beads (0.40 mm in

Table 1. Specific Primers for Amplification of Target Genes from C. albicans SC5314

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<th>Gene name</th>
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* Gene ID number in NCBI.
diameter; Sigma-Aldrich, U.S.A.) in a cell homogenizer. Purification of the target recombinant protein from the supernatant was performed by affinity column chromatography using ProBond™ Nickel-Chelating Resin (Invitrogen) under native conditions. Protein concentration was measured with the BCA assay (BioRad, U.S.A.). Endotoxin Removing Columns (Thermo Scientific, U.S.A.) were used and the endotoxin levels were determined with Limulus Amoebocyte Lysate endochrome (Charles River, U.S.A.) following the manufacturer’s instruction. Using this procedure, endotoxin was reduced to less than 0.1 EU per dose used for subsequent vaccination.

Western Blotting

The V5-6×His-tagged purified proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in a 12% polyacrylamide gel. Proteins were boiled for 5 min in loading buffer. Electrophoresis was run at room temperature (r.t.) for: 80 V, 30 min; 120 V, 1 h. Proteins were stained with Coomassie blue or transferred onto nitrocellulose membranes (Millipore, Bedford, MA, U.S.A.) in Towbin buffer (25 mM Tris-Base, 192 mM Glycine, 20% methanol, pH 8.3) at 5.5 mA/cm² for 15 min by using of Bio-Rad Trans-Blot SD Cell (Hercules, CA, U.S.A.). The membrane was blocked for 1 h in phosphate buffered saline (PBS) (pH 7.4) containing 5% skim milk and washed three times for 10 min in PBS containing 0.05% Tween 20 (PBS-T). The membranes were incubated with mouse monoclonal anti-V5 antibody (dilution 1:5000; Invitrogen) overnight at 4°C. After washing for five times with PBS-T, membranes were incubated with DYLight™800-Labeled antibody to mouse IgG (H+L) (dilution 1:10000; KPL) for 2 h at r.t. After further washing with PBS, membranes were developed with Odyssey (LI-COR, U.S.A.).

Immunoprecipitation and Collection of Blood Samples

Based on Edwards’s study of rAls1p-N and rAls3p-N vaccine⁹ and our preliminary vaccination experiments, the appropriate immunization dose was determined. Ten BALB/c mice (6–8 weeks) were immunized by subcutaneous (s.c.) injection of 20 μg of each purified recombinant target protein mixed with complete Freund’s adjuvant (CFA; Sigma-Aldrich) at day 0, boosted with 10 μg of each recombinant protein in incomplete Freund’s adjuvant (IFA; Sigma-Aldrich) at day 21, and then infected via the tail vein with 1×10⁶ cells of C. albicans SC5314 at day 35. Before challenge, the sera from each mouse were pooled and analyzed subsequently. The control mice were vaccinated with adjuvant alone or together with the tag protein (V5 epitope and 6×His Tag) on the same schedule. Mice treated with saline followed by subsequently lethal dose of SC5314 were used as placebo group. The experiments were carried out at least three times and all sera were stored at −80°C.

All mice were monitored at least twice daily and were euthanized by CO₂ asphyxiation after 15d infection. Distress symptoms such as hair coat appearance, ability to get up and down, activity ability, and weight loss were rated for severity as 0 (in good condition), 1 (mild), 2 (moderate) and 3 (severe). If the mouse had a score of 3 for any distress symptom, it was euthanized by CO₂ asphyxiation. Because this work focused on investigating the effects of vaccination of each recombinant target protein to systemic C. albicans infection, survival and organ fungal burden of mice must be examined. Any intervention to alleviate pain (other than euthanasia) would have obfuscated the results. Therefore, it was required that the experiments after C. albicans infection be carried out without administering anesthetics or analgesics. Yet before the experimental operations including sera pooling, fungal challenge, organ harvesting and passive antiserum transfer, mice were anaesthetized intraperitoneally with 1% pentobarbital sodium (50 mg/kg).

Organ Fungal Burden and Histopathological Assessment

The heart, liver, spleen, lungs, brain, and kidneys were harvested from 5–8 mice of each group at day 5 post infection, weighed, suspended in sterile PBS, homogenized and spread in duplicates on Sabouraud’s agar (SDA) plates. Colonies were counted after incubation at 37°C for 48 h. The results were expressed as Log₆₉ colony formation unit (CFU) per gram of infected organs. The samples were from at least five mice. The experiments were carried out at least three times.

The kidneys were removed aseptically from three mice per group for histopathological examination and were then immersed in 10% (v/v) formalin until examination. Fixed organs were dehydrated in graded alcohol solutions, embedded in paraffin with 3–4 μm-thick sections, and stained with Periodic Acid–Schiff stain (PAS).

Passive Immunization by Antiserum Transfer

BALB/c mice were passively immunized by a single intraperitoneal injection of 1 mL of immuned, control or placebo mouse serum. The injected serum was heated at 56°C for 1 h before the passive transfer experiments in order to inactivate heat-labile nonantibody constituents. On the next day after the serum transfer, mice were challenged with a lethal dose (1×10⁶ cells) of C. albicans SC5314 via the tail vein. Protection capacity against this lethal challenge was evaluated by assessment of the survival and organ fungal burden as described above. All mice were monitored twice daily and were euthanized by CO₂ asphyxiation after 15d infection. The experiments were carried out at least three times.

Serological Analysis

Pooled sera for antibody assays were collected from 5–8 mice per group at day 35 by using a standard enzyme-linked immunosorbent assay (ELISA) assay following standard protocols. Polystyrene microtitre plates (Nunc-Immuno Plates, Nunc, San Diego, CA, U.S.A.) were coated overnight at 37°C with 1 μg of purified rPmt4 protein antigen or with C. albicans SC5314 1×10⁶ live cells in 0.1st carbonate–bicarbonate buffer (pH 9.6) per well. After blocking of nonspecific sites with 5% non-fat milk powder in PBS-T for 2 h at 37°C, 100 μL of serial dilutions of each serum were incubated in the plates for 2 h at 37°C. After extensive washing, bound antibodies were detected by the addition of 100 μL of horseradish peroxidase (HRP)-conjugated goat anti-mouse Immunoglobulin G (IgG) (H+L) (1:250, KPL) or IgM (1:2500, KPL) to each well and incubation for 1 h at 37°C, and then were developed with substrate 3,3′,5,5′-tetramethylbenzidine. The absorbance was measured at 450 nm after the addition of 2 M H₂SO₄ (50 μL per well) to stop the reaction. Background absorbance was subtracted from the test wells to obtain the final optical density readings. The antibody titres were defined as the highest dilution of mouse serum that gave an optical density which was at least twice that of the negative control. All experiments were carried out at least three times.

Phagocyte Killing Assay

In vitro neutrophil-mediated killing was examined as previously reported.¹⁰,²⁹ Neutrophils were isolated from peripheral blood from healthy mice using a Neutrophil Isolation Kit (tbdscience, China). C. albicans
SC5314 was incubated with 10, 20 and 50% (v/v) of the immuned, control or placebo mouse serum in PBS, in a 37°C water bath for 30 min, and washed twice with PBS before use. *C. albicans* cells incubated in PBS without serum were used as the negative control. A total of $4 \times 10^4$ neutrophils were co-cultured with $2 \times 10^4$ serum-treated *C. albicans* cells for 2 h at 37°C in PBS. After incubation, residual neutrophils were lysed. *C. albicans* cells were spread on YPD medium plate, and incubated for 48 h at 37°C. CFU were counted and the percentage of killing was calculated as follows, ($1-$CFU from the plates of *C. albicans* cells with neutrophils/CFU from the plates of *C. albicans* cells only)×100%. The experiments were carried out at least three times.

**Efficacy of Anti-rPmt4p Serum Cooperated with Fluconazole (FLC) against *C. albicans*** A suspension of 100 µL of *C. albicans* SC5314 containing 5×10³ cells/mL in RPMI 1640 was added into 96-well cell culture plates containing 0.25 µg/mL of FLC which is the 80% of minimal inhibitory concentration (MIC₈₀) determined as standard Clinical and Laboratory Standards Institute (CLSI, formerly NCCLS) procedure.³⁰ Then 10% (v/v) of the immune, control or placebo serum from day 35 were added to the wells. Optical density at 620 nm was determined after 48 and 96 h of incubation at 30°C. The experiments were performed at least three independent biological samples in duplicates.

**Detection of Cytokines in Serum by Cytometric Bead Array (CBA)*** Serum of experimental animals was taken at day 35 and analyzed for IL-10, IL-12p70, IL-17A, IL-4, and TNF-α with the CBA Flex Set (BD Biosciences, Mountain View, CA, U.S.A.) by using a BD FACSCalibur™ flow cytometer. The limit of detection was 10 pg/mL. Samples whose cytokine levels were undetectable were assigned the detection limit values for graphing and analyzed with FCAP Array Software (BD Biosciences, Mountain View, CA, U.S.A.). All experiments were performed at least three independent biological samples in triplicates.

**Statistical Analysis** The nonparametric log rank test was used to determine differences in the survival time of the mice. Organ fungal burdens among different groups were compared by using the Mann–Whitney U test for unpaired comparisons, as appropriate. The significant value was set at p<0.05.

**RESULTS**

**Preparation, Purification and Characterization of Recombinant Target Proteins** In order to clone and express 11 recombinant target proteins of *C. albicans* in *S. cerevisiae* diploid strain INVSc1, the corresponding coding sequence was amplified by polymerase chain reaction (PCR) from the genomic DNA of *C. albicans* strain SC5314. Only a single band with an estimated size of each fragment of target gene was found to be present (Supplementary Fig. S1A). Both the double-digestion of the recombined plasmid by the corresponding restriction enzymes and the sequencing showed that target fragment of each gene was correctly cloned in the pYES2/CT vector (Supplementary Fig. S1A; supplemental blast results). The molecular weight of these recombinant fusion proteins including the C-terminal V5–6×His tag was predicted by ProtParam tool (http://www.expasy.ch): rPmt4p, 27 kDa; rAge2p, 21 kDa; rAgp2p, 57 kDa; rAlg2p, 30 kDa; rIf1p, 39 kDa; rMnn46p, 25 kDa; rGpa4p, 54 kDa; rGpa7p, 49 kDa. The identity of the isolated recombinant target proteins were confirmed by Western blotting with mouse monoclonal anti-V5 antibody (Supplementary Fig. S1B).

**Some Recombinant Protein Vaccinations, Especially rPmt4p, Induce Protection against Murine Disseminated Candidiasis** To evaluate the protective effect of the recombinant protein vaccinations, the survival rate was investigated in the mouse model of hematoenously disseminated candidiasis. Mice receiving adjuvant alone (FA in Fig. 1A) or adjuvant together with the V5–6×His tag protein (FA+Tag in Fig. 1A) all died within 9 d post infection. Mice in placebo died within 9 d as well (placebo in Fig. 1A). In contrast, the recombinant rAge2p, rIf11p and rPmt4p vaccinations resulted in significant improvement in survival with survival rate of 30, 40 (p<0.05, Fig. 1A) and 54.5% (p<0.01, Fig. 1A), respectively, at day 15 post-infection. No animals died without human intervention. A total of 420 mice were euthanized. These results suggested that the mice previously challenged with the rAge2p, rIf11p and rPmt4p vaccinations became more resistant to a subsequent lethal dose of *C. albicans*.

As the rPmt4p vaccination resulted in the significant survival rate of 54.5%, which was the highest among 11 recombinant protein vaccinations, we then focus on protection of the rPmt4p vaccination.

In both immunized and adjuvant control mice, the fungi were found in the heart, liver, spleen, lungs, brain, and kidneys (Fig. 1B). But the fungal burden in the heart, liver, brain, and kidneys in the rPmt4p immunized mice were significantly lower than that in the adjuvant alone immunized mice (p<0.05, Fig. 1B), in particular the kidneys and brain showed a 76- and 11-fold reduction respectively (p<0.01, Fig. 1B). All 70 mice used in organ harvesting were euthanized after the organ harvesting. This fungal load reduction suggested that vaccination have slowed down the growth of the infecting *C. albicans* cells.

The severity of tissue lesions estimated by histopathology was consistent with the degree of fungal burden. The inflammatory cells infiltrated into glomeruli in the adjuvant control mice (Fig. 1Ca), but not in the rPmt4p immunized mice (Fig. 1Cb). Hyphae in the renal interstitium cells decreased significantly in the rPmt4p immunized mice (Fig. 1Cd) compared to the control group (Fig. 1Cc). Taken together, these results suggested that the immunization of mice with rPmt4p vaccination effectively protected mice against systemic infection of lethal-dose of *C. albicans* SC5314.

The rPmt4p Vaccination Increases Antibodies in Immune Serum Sera were collected from both the rPmt4p immunized and the adjuvant control mice at day 35. No rPmt4p-specific antibody was detected in the control serum (Fig. 2A). However, high levels of IgG and IgM against rPmt4p were detected in the immunized serum with the titer reaching 1:32000 (p<0.01, Fig. 2A) and 1:4000 respectively (p<0.05, Fig. 2A). In order to explore whether the immunized serum can recognize live *C. albicans* cell, antibodies against live cell were examined. After an exposure to the recombination protein for 35 d, IgG against live cell antigen was identified in the immunized serum with a titer of 1:4000 (p<0.05, Fig. 2B), suggesting that IgG might confer immunity to the mice. But the anti-live cell IgM was not significantly increased in the immune serum compared to that in the adjuvant control mice.
(Fig. 2B). This was expected because the mice haven’t been exposed to the fungal pathogen yet.

**Protection Capability of Passive Transfer of the rPmt4p Immunized Serum** A total of 90 mice used in the survival and 72 mice used in the organ harvesting experiments were all euthanized. As shown in Fig. 3, passive transfer of the rPmt4p immunized serum increased survival rate to 40% and significantly reduced fungal burden in the heart, brain, and kidneys, compared with the control group. These results indicated that the production of antibodies IgG and IgM elicited by immunization with rPmt4p vaccination contributed to protection against systemic *C. albicans* infection.
The rPmt4p Vaccination Increases T-Helper (Th)1/Th2/Th17 Cytokines in Immunized Serum

The effect of rPmt4p vaccination on the secretion of cytokines and chemokines were then evaluated. The levels of the Th1 cytokines interleukin (IL)-12 and tumor necrosis factor (TNF)-α, Th2 cytokines IL-4 and IL-10, and Th17 cytokines IL-17A in the immunized serum were increased compared to those in the control mice, especially IL-12p70 (\(p<0.01\)), IL-17A (\(p<0.01\)) and TNF-α (\(p<0.001\), Fig. 4).

Anti-rPmt4p Serum Enhances Susceptibility of C. albicans to Neutrophil-Mediated Killing

During hematogenously disseminated infection, neutrophils in host can kill C. albicans cells either extracellularly or intracellularly. To define potential interactions of rPmt4p vaccination with host cells, we then investigated the effect of the anti-rPmt4p serum on neutrophil-mediated killing. As shown in Fig. 5, the 10% and 20% of anti-rPmt4p serum significantly increased neutrophil-mediated killing of C. albicans in vitro in comparison with the control serum (\(p<0.01\)). Interestingly, the lower (10%) and intermediate (20%) dose of anti-rPmt4p serum provided superior killing compared to the higher dose (50%).

Anti-rPmt4p Serum Increases the Fungistatic Efficacy of FLC against C. albicans

To evaluate the effect of the rPmt4p immunized serum against C. albicans in vitro, the effect of anti-rPmt4p serum combined with FLC against C. albicans were estimated. In the absence of serum, 0.25 \(\mu\)g/mL of FLC inhibited 42.8% of cells after 96 h of incubation (\(p<0.05\), Fig. 6). In the presence of adjuvant control serum, FLC inhibited 47.6% of C. albicans SC5314 cells (\(p<0.05\), Fig. 6). However, the addition of anti-rPmt4p serum, FLC significantly inhibited the growth of C. albicans SC5314 by 64.5% (\(p<0.01\), Fig. 6). The survival of C. albicans cells treated with anti-rPmt4p serum and FLC was significantly reduced compared to those in the control or the placebo serum combined with FLC (\(p<0.01\); \(p<0.05\); Fig. 6), or even lower than that without serum (\(p<0.05\); Fig. 6). These results indicated that the anti-rPmt4p serum enhanced the inhibition of FLC against C. albicans SC5314.
The passive transfer of the rPmt4p immunized serum further demonstrated by 35–36 Our results suggest that the mechanism of protection rendered by rPmt4p vaccination appears to be attributed, at least in part, to protective antibody response. The anti-rPmt4p serum enhanced the neutrophil-mediated killing of C. albicans and fungistatic efficacy of FLC against C. albicans. We hypothesized that serum factors may contribute to such effects. Our results show that anti-rPmt4p serum produce a wide range of cytokines that might play a role in mediating T-cell activation, including inflammatory cytokines (TNF-α), anti-inflammatory cytokines (IL-10), type 1-inducing cytokines (IL-12), as well as type 2 cytokines (IL-4). Among them, the levels of IL-12 and TNF-α in the immune serum were significantly higher than those in the control group. TNF-α and IL-12 have important roles in the induction of T-cell responses to Candida and host defense against candidiasis. TNF-α mediates pro-inflammatory activities in the early phases of the host response, and the TNF-α−/− mice have increased susceptibility to systemic C. albicans infection.37) IL-12 plays a crucial role in inducing IFN-γ, as well as Th1 responses. The IL-12−/− mice are deficient in IFN-γ production.38) The IFN-γ knockout mice have increased susceptibility to both gastric and systemic candidiasis.39)

Our results showed increased production of inflammatory cytokine IL-12 and TNF-α in response to C. albicans antigens, which was paralleled by a significant increase in the production of the anti-inflammatory cytokine IL-4 and IL-10 in the rPmt4p-immunized serum. The role of inhibitory cytokines, such as IL-10, is crucial in down-regulation of type 1 cytokines (IL-12, IL-18, and IFN-γ), as well as inflammatory cytokines (IL-6 and TNF-α).40) Th17 cells produce proinflammatory cytokines like IL-17A, IL-17F, IL-21 and IL-22, and are involved in the clearance of several extracellular bacteria and fungi.41) The protective roles of IL-17A in a murine model of infection with C. albicans have been demonstrated.42–43) IL-17A-producing γδ-T cells involve in the first line of host defense against C. albicans infection.42) A vaccine that elicited an inflammatory reaction alone might damage the host when the infectious agent was encountered. The existence of a high degree inflammation in vaccinated mice before challenge, as demonstrated by >1000 pg/mL of TNF-α, raises the possibility that the outcome of the rPmt4p vaccination is partly due to the inflammatory response to rPmt4p plus adjuvant rather than induction of acquired immunity. In contrast, a vaccine that elicited some Th2, Th17-type regulatory cytokines could provide a more balanced response. In this study, the production of Th1 cytokines IL-12 and TNF-α, Th2 cytokines IL-4 and IL-10, as well as Th17 cytokines IL-17A in the anti-rPmt4p serum, suggested that rPmt4p vaccination could provide a more balanced response to C. albicans infections. Modulation of putative interactions between Th1/Th2/Th17 cytokines in the context of C. albicans infections has been postulated as having therapeutic potential.

FLC is the most common used antifungal agent. The acquired resistance to FLC in C. albicans has been the major problem in treatment. Combination therapy is the promising means of combating resistance and improving clinical outcome. The present data showed that the anti-rPmt4p serum increased the inhibition of FLC against fluconazole-susceptible C. albicans SC5314. This enhanced activity might reflect the
effect of combining two drugs directed against different targets within the fungus.

In conclusion, among the 11 recombinant proteins, the rPmt4p vaccination conferred significant protection against experimental disseminated candidiasis by inducing effective killing of phagocytosed *C. albicans* in neutrophils, and production of protective antibodies IgG/IgM and Th1/Th2/Th17 cytokines, which support the idea that rPmt4p vaccination could stimulate both cell-mediated and humoral immunity.

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**Conflict of Interest** LY and YJ claim that Second Military Medical University holds patents on the use of rPmt4p as a vaccine against disseminated candidiasis.

**Supplementary Materials** The online version of this article contains supplementary materials. Supplementary Fig. S1 and blast results are available as supplementary materials.

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