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Hybrid phage displaying SLAQVKYTSASSI induces protection against *Candida albicans* challenge in BALB/c mice

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The polymorphic fungus *Candida albicans* (*C. albicans*) can live as an aggressive pathogen and cause many diseases in hosts, for which no effective vaccine exists. The secreted aspartyl proteinase 2 (Sap2) plays a protective role in systemically infected BALB/c mice. Protective cellular immune responses can be preferentially induced when antigens are displayed on small particles. Therefore, the emphasis is placed on developing new phage vaccine to inhibit *C. albicans* infection. In this study, the ability of the hybrid phage displaying the epitope SLAQVKYTSASSI and recombinant protein of Sap2 (rSap2) for inducing immune protective responses against *C. albicans* infection was evaluated by lymphoproliferative assay, to gather cytokine and antibody measurements in BALB/c mice. Our results showed that, strong cellular and humoral immune responses were induced in a mouse model immunized with hybrid phage or rSap2. Furthermore, the protection against lethal challenge with *C. albicans* was observed in mice vaccinated hybrid phage without adjuvant. These findings demonstrate that the hybrid phage displaying the epitope SLAQVKYTSASSI might be a potential vaccine against *C. albicans* infections.

Introduction

C. albicans is an opportunistic pathogen yeast, it is implicated as an agent of diseases in immunocompromised groups, diabetes, and cancer patients.[1,2] *Candida* species are now the fourth most common microbe isolated from nosocomial bloodstream infections in the United States hospitals with a mortality rate around 40%.[3] In addition, carriers of synthesized peptide vaccine are difficult for synthesis, and have safety and solubility issues. Although 2 Candida vaccines were shown to be safe in Phase 1 trials, neither vaccine has been shown to be efficacious in humans.[4,5] Therefore, the emphasis is placed on developing new vaccine to inhibit *C. albicans* infection.

Both basic and clinical studies confirm that T cell-mediated immune responses to *C. albicans* infection, particularly those associated with Th1- and Th17- type immune responses are pivotal for protection against this pathogen.[6-8] The BALB/c mouse is extensively used as an animal model for studies of immunity to *C. albicans* infection.[9,10] Differences in susceptibility to *C. albicans* infection were observed in a variety of different tissues, Th1-mediated immunity was thought to confer the primary protection, particularly for candidiasis.[11-13] Furthermore, Th17 compartment was shown to play a predominant role in mucosal candidiasis.[14-16]

Numerous studies described the presence of Secreted aspartic proteins (Saps) during *C. albicans* infections. Sap2 is produced by *C. albicans* at the early stage of infection, it is well known to degrade many host proteins.[17-20] Sap2 could not only provide essential nitrogen for growth, but also enhance attachment, colonization, and penetration of host tissue by the removal of host barriers.[21,22] The antibody against Sap2 was protective in systemic candidiasis and an adjuvant-free virosomal formulation of a recombinant Sap2 vaccine induced protection in an experimental model of rat vaginal candidiasis.[23,24] These findings led to its evalution as a vaccine antigen where it was demonstrated to have protective efficacy in preclinical animal model of candidiasis. Additionally, we displayed epitope VKYTS of Sap2 on phage and found that the hybrid phage could induce the specific antibody production in mice and recognize the antibody in *C.albicans* infected patients.[25]

To evaluate whether hybrid phage represents a potential novel vaccine candidate without adjuvant against *C. albicans*, we constructed a plasmid pET28a-*Sap2* and examined the humoral and cellular immune responses in BALB/c mice after immunization with hybrid phage displaying epitope SLAQVKYTSASSI and rSap2, and assessed the protective immunity induced by the stimulants. In this paper, we describe that the use of the phage for delivery of *C. albicans* Sap2 epitope for effective immunization of BALB/c mice, and the hybrid phage displaying SLAQVKYTSASSI has a great potential as a vaccine to induce strong Th1 and Th17 response without adjuvant.

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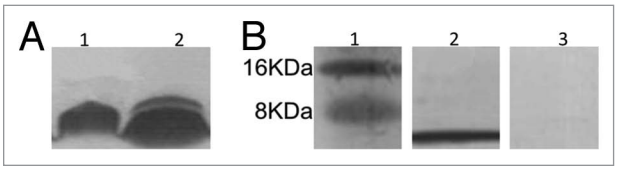

Figure 1. SDS-PAGE for hybrid phage and WT phage. (**A**) Lane 1, WT phage, lane 2, Hybrid phage. (**B**) Western blot assay for hybrid phage with sera. Lane 1, Marker; Lane 2, Sap2 -immunized mice serum; lane 3, non-immunized mice serum.

Results

Antibody response against hybrid phage and rSap2

The recombinant phagemid pfd8SHS was transformed into *E. coli* TG1, and produced hybrid phage displaying epitope SLAQVKYTSASSI. The phage displaying epitope SLAQVKYTSASSI was analyzed by SDS-PAGE (**Fig. 1A**) and reacted with anti-sera of rSap2 (**Fig. 1B**). Sequence analysis confirmed that the Sap2 gene was cloned into pET28a successfully. In addition, rSap2 was analyzed by SDS-PAGE, a 43 kDa protein band was visible (**Fig. 2A**), and it could be recognized using the sera from mice immunized with hybrid phage and rSap2 (**Fig. 2B**). The total IgG antibodies against hybrid phage and rSap2 were measured in sera of the immunized mice by ELISA. The results indirectly showed that mice vaccinated with the hybrid phage and rSap2 displayed a significantly high level of total IgG compared with mice vaccinated with TE alone ($P < 0.01$) (**Fig. 3**), and demonstrated that the hybrid phage and rSap2 can induce specific antibody production in BALB/c mice.

Splenocytes proliferation induced by rSap2 and hybrid phage

The splenocytes were prepared from the immunized mice to assess the proliferative immune responses. As shown in **Figure 4**, compared with those of mice immunized with TE, the splenocytes from all of the mice immunized responded vigorously to rSap2 ($P < 0.01$). Although no difference was detected among the 3 immunized groups, significant difference compared with TE injected group was found ($P < 0.01$).

Cytokines levels in splenocytes culture supernatant

To assess the development of cell-mediated immunity, splenocytes of immunized mice, or TE-injected mice were restimulated in vitro by immunogen for 48 h individually. As shown in **Figure 5**, IFN-γ, IL-2, IL-12, and IL-17 production were significantly higher in immunized animals as compared with the control group ($P < 0.05$). There was no statistically significant difference in the IL-4 and IL-10 production as compared with the control group. Our results showed that both phage displaying epitope and rSap2 could induce Th1- and Th17-type cytokines gene expression.

Cytokines gene expression in spleens

RT-PCR analysis of cytokines was performed on cDNA generated from the total RNA of spleens individually taken from mice. The primers used are shown in **Table 1**, Th1- and Th17-type cytokines, IFN-γ, IL-2, IL-12, and IL-17 were upregulated compared with the control group ($P < 0.01$) (**Fig. 6**). However, the level of IL-4 and IL-10 in WT phage, hybrid phage, and rSap2 immunized mice were similar to TE-injected mice.

Protection against challenge by intravenous inoculation of *C. albicans*

To determine the *C. albicans* colonization in tissues, 3 mice in each group were challenged by inoculating 10^6 *C. albicans* and sacrificed on day 7 post-challenge, and their kidneys and spleens were collected for analysis. In **Figure 7**, higher levels of *C albicans* colonization were detected in the kidneys and spleens of the mice immunized with WT phage or TE, whereas the *C. albicans* colonization was very low in the mice immunized with rSap2 or hybrid phage. Compared with those of control group, little *C. albicans* colonization was detected in the kidneys of mice immunized with hybrid phage ($P < 0.01$). To evaluate the potency of hybrid phage against lethal *C. albicans* challenge, the immunized mice were challenged with 10^7 *C. albicans* on day 10. Starting from day 2 post-challenge, the mice immunized with antigens started dying. The survival rates in the hybrid phage, rSap2, WT phage, and TE groups on 10 d post-challenge were 50%, 62.5%, 8.75%, and 0% respectively (**Fig. 8**).

Disussion

Altough humoral immune response is critical for protection against candidiasis, several studies showed that cell-mediated immunity also play important roles. Recent studies have focused on the ability of vaccines to polarize the activation of specific T helper cell populations (e.g., Th1, Th2, and Th17) against fungal infections,[26] some evidences also showed that Th1 cells

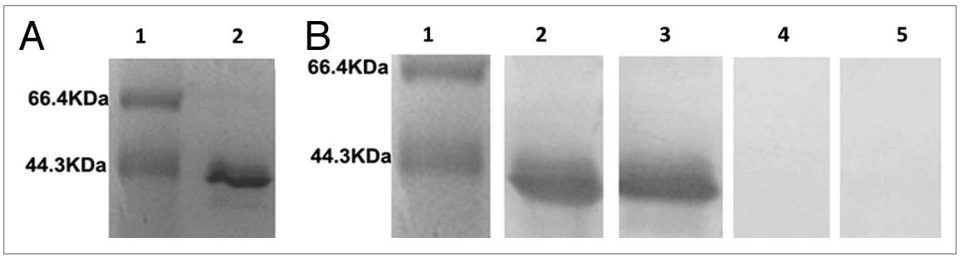

Figure 2. rSap2 recombinant protein and western blot assay. (**A**) rSap2 protein purified by Ni^+ affinity column. Lane1, Marker; Lane2, rSap2. (**B**) Western blot assay for rSap2 with immunized or non-immunized sera. Lane 1, Marker; Lane 2, Sap2 -immunized mice serum; lane 3, hybrid phage immunized mice sreum; lane 4, WT phage immunized mice sreum; Lane 5, non-immunized mice serum.

conferred resistance to most extracellular microbes, including *C. albicans*.[27-29] Furthermore, emerging data indicated that the newly defined Th17 cells played the predominant role in mucosal candidiasis.[30] The protective immunity against *C. albicans* was described in murine studies. We analyzed T cell immunity by measuring cytokine responses to rSap2 and the phage displaying SLAQVKYTSASSI and found that both were immunogenic.

Sap can modify the epithelial cytokines expression in a vitro model of vaginal candidiasis,[31] and Sap2 knockout mice reduces damage to the tissue and has a significantly reduced potential to the cytokines expression.[32] On the other side, rSap2 can induce both humoral immune response and cellular immune response, which may play an important role in the anti-Candida protection.[24] In this study, rSap2 was demonstrated to induce pro-inflammatory cytokines such as IFN-γ and IL-2 which produce stimulation of Th1 cells and CD8 T cell, and IL-17 which plays a role in *C. albicans* infection. Moreover, a slight increase in Th2 cytokines was observed following Sap2. Therefore, we hypothesized that suppression/immunoregulation by Th1 cytokines might exist. Vilanova et al. reported that protection against systemic candidiasis in mice immunized with Sap2 was antibody mediated and antibodies also were reported to mediate anti-*Candida* protection in animals immunized with almost all reported experimental vaccine.[4,23,33] Therefore, the protection mediated by Sap2 might refer to antibody, Th1- and Th17-type cytokines.

T cell epitope is located at the C-terminal region of Sap2, which (e.g., SLAQVKYTSASSI) might stimulate the proliferation of splenocyte and then induced the production of cytokines.[34] In *C. albicans* infection, IgG identified epitope in amino acid position 386–390 (VKYTS) is associated with antibody production and survival.[35] In previously study, a hybrid phage displaying VKYTS was produced, which was found to have the ability to induce and recognize anti-Sap antibody and could be used to detect *C. albicans* infection. In contrast to epitope VKYTS, the epitope SLAQVKYTSASSI could both induce cellular and humoral immunity to make it more adaptable for vaccine research.

Previously we reported that hybrid phage could induce Th1 immune response which was related to protection.[36] To further evaluate the possible immunomodulatory actions of hybrid phage, cytokines production and cell proliferation by murine splenocytes were examined in the presence of these stimuli. In this study, our data showed that hybrid phage displaying SLAQVKYTSASSI was same as rSap2 which could cause a significantly improvement in stimulation of immune responses. Hybrid phage and rSap2 stimulated splenocytes proliferation, induced Th1 and Th17 response pattern, and enhanced secretion of IL-2, IL-12, IFN-γ, and IL-17. Moreover, serum antibody of mice immunized with hybrid phage could recognize rSap2. Therefore, these findings suggested that hybrid phage had the potential in vaccine production, which probably played a major role in humoral immune response and cell-mediated immunity response.

Phage displaying SLAQVKYTSASSI contains T-cell and B-cell epitopes, which does not need adjuvant to enhance the role, and the hybrid phage delivery induced balanced Th1 and Th2

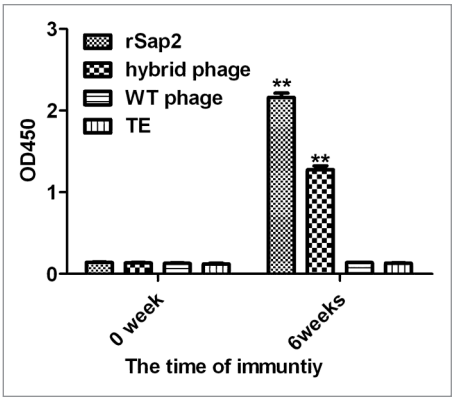

Figure 3. IgG determination by ELISA. Serum IgG responses in mice vaccinated with hybrid, rSap2, WT phage on weeks 0 and 6. Sera were collected on 1 d prior to each immunization, and tested by ELISA using rSap2. The titer was given as the reciprocal of the highest dilution with an OD450 that was 2.5-fold greater than the OD of TE treated mouse sera at the same dilution.

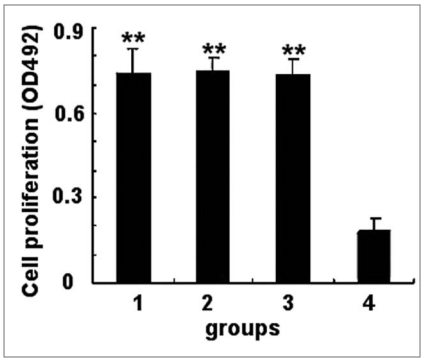

Figure 4. Splenoccytes proliferation measured by MTT. Cells were seeded into a 96-well plate with the density of 5×10^3 cells per well and cultured with stimulus at 37 °C. MTT assay was performed after 72 h. The data shown are means ± SD of 3 independent experiments. Group 1, rSap2 immunized mice; group 2, hybrid phage immunized mice; group 3, WT phage immunized mice; group 4, TE injected mice. **Statistically significant ($P < 0.01$).

responses with a potent Th1 response. Similar patterns were observed in our previous studies with the phage displaying epitope LKVIRK.[37] In addition, we showed for the first time that the hybrid phage induced strong Th17 response. Therefore, phage seems to be a suitable carrier of an epitope. rSap2 is a good vaccine candidate, which mediates protection by specific antibody and cytokines, however it needs immune adjuvant. The specific immune response, the ability to recruit helper T-cells and the unnecessity for external adjuvant suggest phage may be an inexpensive and particularly useful platform for epitope vaccine design. We also note that, although the mechanistic basis for phage responses is currently unknown, no adverse effects to phage vaccination have been observed in many preclinical studies performed in animal models.[38]

Our results showed that the hybrid phage immunization had the same protection as recombinant protein immunization. The

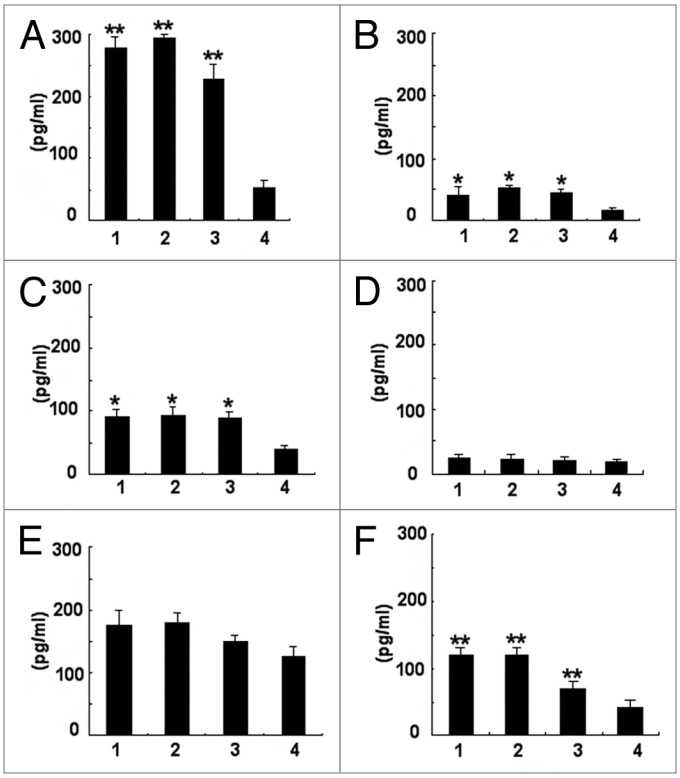

Figure 5. Quantitative sandwich ELISA assay for cytokines expression in splenocytes culture supernatant. (**A**) IFN-γ secretion; (**B**) IL-2 secretion; (**C**) IL-12 secretion; (**D**) IL-4 secretion; (**E**) IL-10 secretion; (**F**) IL-17 secretion. For (**A–F**), 1, rSap2 immunized mice; 2, hybrid phage immunized mice; 3, WT phage immunized mice; 4, TE injected mice. The data shown are means ± SD of 3 independent experiments. *Significant ($P < 0.05$), **Statistically significant ($P < 0.01$).

Table 1. Primer sequences used in polymerase chain reaction to study the gene expression of cytokines

Target	Primers sequence
β-actin	Sense 5′-AGGGAAATCG TGCGTGACAT CAAA
	Antisense 5′ -ACTCATCGTA CTCCTGCTTG CTGA
IL-2	Sense 5′ -TTGATGGACC TACAGGAGCT CCTGAGCA
	Antisense 5′-AGAGAGCCTT ATGTGTTGTA AGCAGGAGG
IL-4	Sense 5′-TGACGCACAG AGCTATTGAT GG
	Antisense 5′-ATGATGCTCT TTAGGCTTTC CAG
IL-10	Sense 5′-GGACAACATA CTGCTAACCG ACTC
	Antisense 5′-AAAATCACTC TTCACCTGCT CCAC
IL-12	Sense 5′-CCACTCACAT CTGCTGCTCC ACAAG
	Antisense 5′-ACTTCTCATA GTCCCTTTGG TCCAG
IFN-γ	Sense 5′-AGGAACTGGC AAAAGGATGG TG
	Antisense 5′-GTGCTGGCAG AATTATTCTT ATTG
IL-17	Sense 5′- CTCAAAGCTC AGCGTGTCCA A
	Antisense 5′-TCATGTGGTG GTCCAGCTTT C

adjuvant-free phage decorated epitope induced specific antibody response and cell response, as well as provided protection to mice against very high doses of *C.albicans* challenge. After first challenge, results showed that hybrid phage immunized group and rSap2 immunized group had significant reduction in the number of *C. albicans* at day 7. After the second survival challenge, mice were inoculated with 10^7 *C. albicans*, and it was obvious that immunization with hybrid phage or rSap2 could induce immune response and confer protection against lethal challenge in a mouse model, more important, the characteristic of hybrid phage vaccination was that it could directly be applied in the animals without adjuvant. Therefore, phage-displaying epitope could avoid the problems of adjuvant of recombinant proteins.

Finally, such phage displaying epitope vaccine could be targeted to antigen-presenting dendritic cells by displaying a epitope on the casipd of phage, further stimulating the immunity without adjuvant. In this paper, the hybrid phage displaying the epitope SLAQVKYTSASSI could induce efficient humoral and cell-mediated immune responses, increase survival rate after candidiasis challenge, it may be a potential vaccine against *C. albicans* infections. With the recent data, we could currently develop the phage vaccine platforms, not only to defend against *C.albicans* infection, but also to generate efficacious vaccines against other infectious agents.

Materials and Methods

Strains, phage, and animals

Phagemid pfd8SHS which was propagated in *Escherichia coli* (*E. coli*) strain TG1 and wild-type phage (WT phage) used in this work were stored in our laboratory. Female BALB/c mice, between 6 and 8 wk of age, were purchased from the Specific-Pathogen-Free Animal Facility of Jilin University, China.

Ethics statement

This study was performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of China Association for Laboratory Animal Science. The Ethics Committee of Northeast Normal University approved all animal care and protocols. All sacrifices were performed under pentobarbitone anesthesia, and every effort was made to minimize animal suffering.

Construction of recombinant phagemid pfd8SHS

The construction of recombinant phagemid pfd8SHS and the production of hybrid phage were performed as described previously.[25] Briefly, 2 synthesized complementary oligonucleotides encoding the epitope SLAQVKYTSASSI were ligated into *Sac*II-*Bst*BI digested phagemid pfd8. *E. coli* TG1 transformed by the recombinant phagemid with correct insertion was grown in LB liquid medium supplemented with ampicillin. Hybrid phage was secreted into the culture with the help of WT phage. Then, the culture supernatants containing phage particles was pooled and purified in polyethylene glycol-6000 (final concentration 5% polyethylene glycol-6000, 0.5 M NaCl) by 2 consecutive precipitations for 12 h, and then the phage pellet was

resuspended in 2 ml TE (1.0 mM EDTA, 0.01 M Tris–HCl, pH 8.0). The amount of phage produced was estimated by spectrophotometry, where an OD of 1.0 at 270 nm was assumed to correspond to a concentration of 1/3.84 μg/μl.

Construction of plasmids for production of Sap2

The genomic DNA was prepared by mechanical disruption with glass beads. *C. albicans* ATCC10231 were grown at 28 °C for 18 h and the microorganisms were collected. After 2 washes with 0.9% NaCl, the cells were disrupted with glass beads. Nested PCR was used to amplify Sap2 gene. The PCR product was digested with *E*coRI/*X*hoIand cloned back into the vector pET-28a. The pET28a–*Sap2* recombinant plasmid was transformed into *E. coli* BL21. Expression of recombinant Sap2 (rSap2) was induced by 1 mmol/l IPTG, the protein was extracted in denaturing conditions according to the protocol and its purification was performed by immobilized-metal affinity chromatography with Ni-NTA agarose beads following the manufacturer's instructions (GE).

Immunization of mice

All experiments were done in compliance with international legal and institutional guidelines. Mice were divided into 4 groups, with 27 mice per group. The mice were immunized on weeks 0, 2, 4, 6 with (1) 25 μg rSap2 coupled with Freund's complete adjuvant (FCA), (2) 25 μg hybrid phage particles in 200 μl TE, (3) 25 μg wild-type phage particles in 200 μl TE, and (4) 200 μl TE as negative control. One week after the fourth injection, antibody responsed against rSap2 protein were assessed in the sera of vaccinated and control mice by western Blot and ELISA.

Cell proliferation

Cell proliferation was assessed using MTT assay. One week after the fourth immunization, the animals in each group were euthanized, spleens were removed, and a single-cell suspension was prepared by passage through a 100-gauge wire mesh sieve. The cells were cultured in triplicate wells of flat-bottomed 96-well plates at a concentration of 2.5×10^4 cells/ml, and incubated with medium alone or in medium containing a pool of rSap2 or hybrid phage (25 μg/ml). Twenty μl MTT (5mg/ml pH 7.4) was added after 72 h and incubated for 4 h. After removal of MTT, the formazan crystals was solubilized in DMSO (150 μl/well) and measured using a Microplate Reader (Thermo, USA) at 492 nm.

Measurement of cytokines in culture supernatants

Levels of cytokine in culture supernatants were measured by ELISA according to the manufacturer's instruction (Wuhan Boster Biological Technology, LTD.). Splenocytes of immunized or control mice were prepared as described above. An amount of 5×10^6 splenocytes/ml was cultured in a final volume of 200 μl in 96-well flat-bottom plates in the presence of the relevant stimulants. Culture supernatants were collected after 48 h incubation to estimate cytokines production. The quantity of each cytokine produced in culture supernatant was obtained to determine a standard curve.

RNA extraction, cDNA synthesis, and RT-PCR

One week after the last immunization, spleens were aseptically dissected, sectioned into equal halves and transferred to 1.5 ml

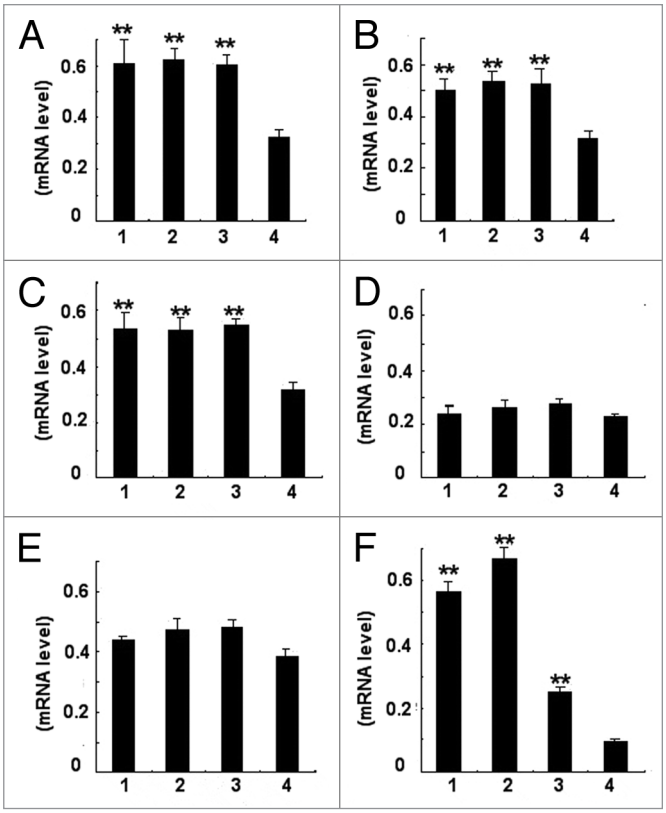

Figure 6. Photodensitometric analysis of PCR products according to RT-PCR assay for cytokines. The histogram showed the ratio of the intensity of cytokines bands to that of β-actin bands. (**A**) IFN-γ mRNA/β-actin mRNA; (**B**) IL-2 mRNA/β-actin mRNA; (**C**) IL- 12mRNA/β-actin mRNA; (**D**) IL-4 mRNA/β-actin mRNA ; (**E**) IL-10 mRNA/β-actin mRNA; (**F**) IL-17 mRNA/β-actin mRNA . For (**A–F**), 1, rSap2 immunized mice; 2, hybrid phage immunized mice; 3, WT phage immunized mice; 4, TE injected mice. The data shown are means ± SD of 3 independent experiments. **Statistically significant ($P < 0.01$).

polypropylene tubes and frozen on ice. Trizol reagent was used for the extractions of RNA as the manufacturer's instructions (Invitrogen). Reverse transcription reactions for first strand cDNA synthesis was also performed according to the manufacturer's protocol (Promega). The amplification reaction and primers used were described previously according to Liu (IL-2, IL-4, IFN-γ), Wang (IL-10, IL-12), and Wang (IL-17) (**Table 1**).[39-41] The PCR products were then separated by electrophoresis in 1% agarose gels containing 1% ethidium bromide. Densitometric analysis of the bands was performed using the Gel Imager Program. The equivalence in intensity between each cytokine and β-actin indicates the relative concentration of mRNA.

Immunization and challenge

Mice were immunized 3 times. Two weeks after the final immunization, 3 mice of each group were inoculated with 10^6 *C. albicans,* 7 d after the infection; each of the challenged mice was sacrificed to count the number of *C. albicans* that migrated to kidney and spleen. Eight mice of each group were inoculated with 10^7 *C. albicans,* and repeat this sample about 2 times. The number of mice that died in 10 d post-infection was counted.

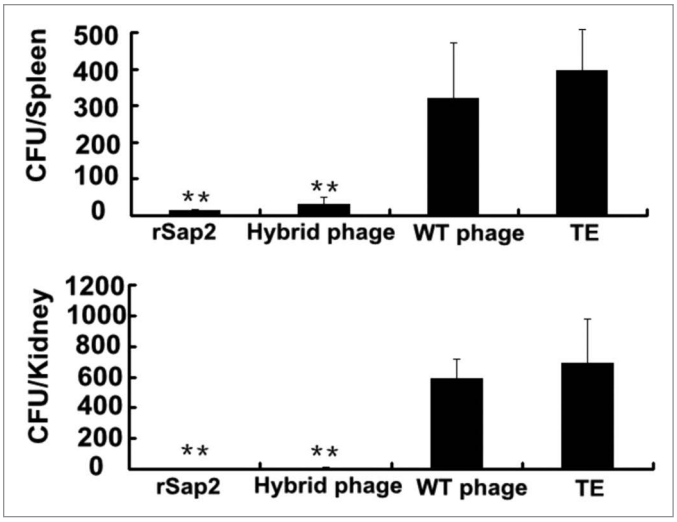

Figure 7. Decreased colonization of *C. albicans* in kidneys and spleens from mice immunized with hybrid phage or rSap2 or wild phage. The numbers of *C. albicans* CFU recovered from kidneys and spleens of mice treated with three injections administered with a period of 2 wk, and infected with 10^6 *C. albicans* 2 wk after the third immunization. The results shown indicate the mean value ± standard deviation (SD) of each group. The significance of the results between immunized hybrid mice and each control. (*$P < 0.05$, **$P < 0.01$).

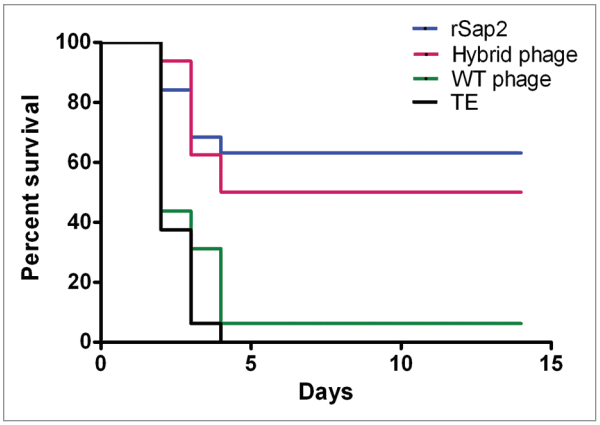

Figure 8. Percent survival in mice immunized with different immunogens. Eight mice of each group were inoculated with 10^7 *C. albicans* after the third immunization and repeat this simple about 2 times. The survival was evaluated for 10 d after challenge.

Statistical analysis

The Student *t* test was used to determine statistical significance between cytokine production and splenocytes proliferation of each immunized group and control group. The criterion for statistical significance was $P < 0.05$.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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