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Design of a mimotope-peptide based double epitope vaccine against disseminated candidiasis

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ABSTRACT

Hematogenously disseminated candidiasis in humans is the third leading cause of nosocomial bloodstream infections in the US. There is no FDA approved antifungal vaccine or prophylactic/therapeutic antibody for use in humans. We first reported novel synthetic peptide and glycopeptide vaccines against *Candida albicans* cell surface epitopes that protect mice against disseminated candidiasis. We showed that antibodies specific for the peptide Fba (derived from *C. albicans* cell surface protein fructose bisphosphate aldolase) or for *C. albicans* cell surface glycan epitope β-1, 2–mannotriose [β-$(Man)_3$]) are both protective. This is an important step forward in vaccine design against disseminated candidiasis in humans. However, given the complexity of oligosaccharide synthesis, in this study we performed a new strategy for use of peptide mimotopes that structurally mimic the protective glycan epitope β-$(Man)_3$ as surrogate immunogens that substitute for the glycan part of glycopeptide [β-$(Man)_3$-Fba] vaccine. All five selected mimotopes are immunogenic in mice and three mimotopes were able to induce protection in mice against disseminated candidiasis. Furthermore, immunization with three mimotope-peptide conjugate vaccines was also able to induce specific antibody responses, and importantly, protection against disseminated candidiasis in mice. Therefore, our new design of a mimotope-peptide based double epitope vaccine against candidiasis is a potential vaccine candidate that is economical to produce, highly efficacious and safe for use in humans.

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1. Introduction

Hematogenously disseminated candidiasis is the third leading nosocomial bloodstream infection in the US. Despite the availability of appropriate antifungal therapy, crude mortality in the last decade has remained high, ranging from 36 to 90% [1–4]. As there is no approved antifungal vaccine for use in humans and significant therapeutic challenges remain, disease prevention through active and passive immunization strategies has become critically important [5–7].

Synthetic peptide and glycopeptide vaccines against *Candida albicans* cell surface epitopes have been successfully designed in our lab to protect mice against disseminated candidiasis [8–10]. We showed that antibodies specific for the peptide Fba (derived from *C. albicans* cell surface protein fructose bisphosphate aldolase) and peptide Met6 (derived from *C. albicans* cell surface protein β-1, 2–mannotriose [β-$(Man)_3$]) that comprise the glycopeptide conjugate vaccine are each capable of protecting the animals [8,11–13]. We conclude that peptide Fba, which was initially used as an effective carrier for the glycan epitope in the glycopeptide vaccine formulation [8], is actually a good candidate by itself for vaccine development against candidiasis. Therefore, a major breakthrough is that our glycopeptide vaccine β-$(Man)_3$-Fba is able to induce dual antibody-dependent protective immunity. Such a conjugate would provide dual immune recognition to help ensure protective immunity even against mutant strains of the fungus that may have lost one of the two epitopes. These findings represent unique steps forward in the development of a vaccine for human use. However, given the complexity of oligosaccharide synthesis, the prospect of finding peptides that structurally imitate the protective glycan epitope β-$(Man)_3$ is of great interest as it may greatly simplify vaccine production. In this study, a new strategy used a panel of novel peptide mimotopes as surrogate immunogens that substitute for the β-$(Man)_3$ of the β-$(Man)_3$-Fba glycopeptide vaccine. The glycan epitope β-$(Man)_3$ is apparently expressed by all *C. albicans* isolates, and by several *Candida* species of medical significance such as

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C. tropicalis, C. glabrata, C. guilliermondii and *C. lusitaniae*. The peptide part of the vaccine, Fba peptide is expressed at 100% homology by *C. albicans* and at ~85–91% homology by other *Candida* species. The newly designed mimotope-peptide vaccine is expected to maintain the protective duality of the original glycopeptide vaccine against multiple *Candida* species. Peptide mimotopes were selected by B6.1, an IgM monoclonal antibody (MAb) specific for β-$(Man)_3$ [14,15], against the J404 nonapeptide library. Competition ELISA results demonstrated that five synthetic mimotopes were able to inhibit the binding of glycan specific MAbs to its original glycan epitope β-$(Man)_3$. Furthermore, by dendritic cell (DC) based immunization approach, vaccination with each of three synthetic mimotope peptides induced good antibody production and protection against disseminated candidiasis in mice. Although this vaccine approach would not be generally applicable to humans, it allows us to perform rapid throughput evaluation of candidate short peptides. Each of selected protective mimotopes could serve as a peptide mimotope in place of the carbohydrate moiety in the glycopeptide-TT vaccine formula, which is suitable for human use in further studies [10].

Finally, immunization with the new mimotope-Fba conjugate vaccines provides the same effective protection comparable to the β-$(Man)_3$-Fba conjugate in mice against the disease. This represents a significant step forward in the development of a simple peptide vaccine that protects against human candidiasis.

2. Meterials and methods

2.1. J404 nonapeptide PDPL

The J404 PDPL used in our studies was constructed by Dr. James B. Burritt and is described elsewhere [16–18]. The library displays random nonamer peptides from the N-terminal portion of the pIII capsid protein of kanamycin-resistant, filamentous bacteriophage M13KBst. The J404 library contains an estimated 5×10^8 different nonapeptides at high titer (1×10^{13} pfu/ml).

2.2. Antibodies

Anti-*Candida* IgM MAb B6.1 and IgG3 C3.1 were isolated as previously described [15] and produced in serum-free medium (BG 101 Liquid Kit; Irvine Scientific, Santa Ana, CA). The Abs were concentrated by ammonium sulfate precipitation and exhaustively dialyzed against Dulbecco's PBS (DPBS) (Sigma, St. Louis, MO). Anti-GBS IgM MAb S10 [19], which is specific for the GBS Ibc protein, were prepared as mouse ascites, and the IgM fraction was isolated by distilled water dialysis.

2.3. Candida strains and culture conditions

C. albicans SC5314 (ATCC MYA-2876), was grown as stationary-phase yeast cells (24 h cells) in glucose-yeast extract-peptone (GYEP, 2% glucose, 1% peptone, 0.3% yeast extract) broth at 37 °C, washed and suspended to the appropriate cell concentration (5×10^6/ml, 1×10^6/ml or 5×10^5/ml) in Dulbecco's PBS (DPBS; Sigma), and used to infect mice intravenously (i.v.) as described [15,20]. *C. albicans* strain SC5314 was also used for serum antibody absorption, immunofluorescence staining and flow cytometric analysis.

2.4. Mice

BALB/c female mice (National Cancer Institute Animal Production Program, Frederick, MD) 5–7 weeks old were used throughout. Mice were always maintained in our AAALAC–certified animal facility and all animal experiments were done in accordance with a protocol approved by the Institutional Animal Care and Use committee (IACUC) at LSU Health Sciences Center (LSUHSC).

2.5. Synthetic peptide, mimotope-peptide and glycopeptide vaccines

14-mer peptide Fba is derived from the N-terminus of *C. albicans* cell wall protein fructose-bisphosphate aldolase (Fba). Fba peptide and five mimotopes that structurally mimic the protective *C. albicans* cell surface glycan β-$(Man)_3$, PS2, PS31, PS28, PS55 and PS76 were produced commercially (GenScript). Mimotope-Fba conjugates and glycopeptide β-$(Man)_3$-Fba conjugate were kindly provided by Dr. David Bundle (Alberta University, Canada).

2.6. Isolation and culture of dendritic cells (DCs) from mouse bone marrow

Dendritic cells (DCs) were generated from mouse bone marrow by a previously described method [8,21]. Briefly, donor mice were euthanized by CO_2 asphyxiation, their long bones and tibias were aseptically removed, bone marrow was flushed from the bones by forcibly injecting several ml of RPMI-1640 and clumps were removed or dispersed by gentle pipetting through a sterile 70-mm cell strainer. Red blood cells were lysed (ACK lysing buffer, 0.15 M NH_4Cl, 1.0 mM $KHCO_3$, 0.1 mM EDTA) for 4 min and the remaining bone marrow cells were suspended in complete medium [CM, RPMI-1640 supplemented with 10% FBS (FBS), 2 mM L-glutamine, 1% of nonessential amino acids and 100 units/ml penicillin and 100 μg/ml streptomycin], adjusted to 2×10^5 cells per ml plated in 6-well plates at 5 ml per well and cultured for up to 9 days in the presence of 40 ng/ml of rmGM-CSF and rmIL-4 (R&D Systems) at 37 °C, 5% CO_2. On days 4 and 7 of culture, the same amount of fresh GM-CSF and IL-4 was added to the wells.

2.7. Active immunizations with peptide pulsed dendritic cells

All active vaccinations were conducted as previously described [8,13,22]. DCs were pulsed *in vitro* with Fba peptide, mimotope, mimotope-Fba conjugate or β-$(Man)_3$-Fba conjugate vaccine as described before with minor modification by totally removing CFA for the last booster [8]. Briefly, DCs in culture were pulsed with the peptide antigen (1 μM) on day 6. On day 7, PGE_2 (10^{-7} M) was added along with LPS (2 μg/ml, Sigma) for 24 h to induce DC maturation. On day 9, antigen-pulsed DCs were washed extensively and 5×10^5 in 200 μl DPBS were given by intraperitoneal (i.p.) route as the priming dose to mice. The mice were boosted i.p. at day 14 (1st booster) and day 28 (2nd booster) with fresh antigen-pulsed DCs without adjuvant.

2.8. Immunofluorescence microscopy

Distribution of the glycan epitope β-$(Man)_3$ on *C. albicans* SC5314 yeast cells surface was determined by indirect immunofluorescence. C. albicans hydrophilic stationary-phase yeast were washed four times in cold DPBS and suspended in cold DPBS at $5 \times 10e6$ cells/ml. Two hundred microliters of MAb B6.1 or C3.1 (at 4 μg/ml of DPBS), or 200 μl of each 1:50 diluted immune serum sample was added to a pellet of *C. albicans* yeast cells (5×10^6), which was prewashed with DPBS three times. The yeast cells were suspended in the antibody preparation and incubated while shaking by rotation at room temperature (RT, 22–24 °C) for 1–2 h. After incubation, the yeast cells were washed with DPBS three times, suspended in 200 μl of fluorescein-labeled goat anti-mouse IgM or anti-mouse IgG (Sigma) (stock solution, 1 mg/ml; working solution, 20 μg/ml of DPBS) and incubated at RT described above for 0.5 h. The yeast cells were then washed with DPBS three times

and suspended in 200 μl of DPBS. The cells were observed by confocal microscopy (LSM 510, Zeiss) and photographed (Tmax ASA 400 film; Kodak). The distribution of the glycan β-$(Man)_3$ epitope on the yeast cell surface bound by antibodies in immune sera was compared to that obtained with yeast cells fluorescently stained for detection of the MAbs B6.1 and C3.1 epitope as described previously [23].

2.9. Fungal challenge dose and assessment of protection

For infection, two weeks after the last antigen-pulsed DC administration, immunized or control mice were infected by the intravenous (i.v.) route with a lethal dose of live *C. albicans* SC5314 yeast cells (5×10^5 in 0.1 ml of DPBS) as described before [24]). Passively immunized mice (see below) also received the same lethal dose of yeast cells. Protection was evaluated by monitoring animal survival for up to 80 days and by quantifying the number of CFU (mean ± SE) per kidney pairs [15,25,26]).

2.10. Passive transfer of MAbs or immune sera by intraperitoneal (i.p.) route

Sera pooled from mice vaccinated with either mimotope alone or mimotope-Fba were examined for their antibody contents by ELISA as described above. Passive immunization was performed by single i.p. administration of 0.5 ml of pooled sera of immunized mice, or normal mouse sera from naïve mice 4 h prior to i.v. challenge with a lethal dose of *C. albicans* SC5314 live yeast cells. Infected mice were sacrificed when they became listless and would no longer eat or drink and their kidneys were assessed for CFU as before. All mice were sacrificed on day 60 or 80 post-challenge.

2.11. Statistical analysis

Survival times were statistically evaluated by Kaplan–Meier (GraphPad Prism, version 7), and statistical significance was subsequently calculated for each preset time point of analysis. Two tests were utilized to evaluate differences in survival: the log-rank test was used to assess the course of survival, and ×2 analysis was used to determine the significance of endpoint differences. Student's *t* test was used to analyze differences in the number of colony-forming units (CFU) between groups.

3. Results

3.1. Peptide mimotopes selected by MAbs B6.1 against the J404 nonapeptide library

Bacteriophage display peptide libraries (PDPL) were used to identify peptides that structurally mimic the β-$(Man)_3$ epitope. The PDPL library, termed J404, displays random 9-mer peptides from the ends of the pIII capsid protein of the filamentous bacteriophage M13 [16–18,27] and contains 5×10^8 different nonapeptides at high titer (1×10^{13} pfu/ml). Clones were selected from PDPL that specifically combined with MAb B6.1, which is specific for the β-$(Man)_3$ epitope [15,28]. The peptide sequences of selected clones were deduced by DNA sequencing of the nonapeptide coding region in the genome of each phage clone. Manipulation of the nonapeptide PDPL and appropriate *E. coli* K91 host cells were as described before [17], with some modifications that we have found to be essential for success, that is, pre-adsorbing the PDPL with an irrelevant IgM-coated matrix. The irrelevant IgM was designated S10 and is specific for a protein antigen of group B streptococcus [19,29]. Our results showed that peptide clones specific for the combining site of MAb B6.1 were isolated successfully by three rounds of MAB B6.1 affinity selection & amplification. We proceeded with DNA sequencing and western blot analysis on the third selection pool of phage (data not shown). Our results are shown in Table 1 indicating that the MAb B6.1-specific clones (n = 54) were represented by five unique nonapeptide displays in the PDPL.

3.2. Synthetic mimotopes inhibit the binding of MAbs B6.1 (IgM) and C3.1 (IgG3) to its original glycan epitope β-1, 2–mannotriose [β-$(Man)_3$]

Five peptide mimotopes (PS76, PS31, PS2, PS28 and PS55) are listed in Table 1. When the synthetic mimotopes were employed as inhibitors for MAb B6.1 in competitive ELISA, they were able to inhibit binding of the MAb B6.1 to the original target β-$(Man)_3$ epitope. We also examined an additional MAb C3.1, which has identical specificity as MAb B6.1, except that MAb C3.1 is an IgG3 isotype [20]. MAb C3.1 also protects mice against experimental disseminated and vaginal candidiasis [14].

Maximum inhibitory activity was achieved with mimotopes PS31, PS2 and PS28 regarding the binding of both MAbs B6.1 and C3.1. The analyses also allowed us to determine the relative binding affinities of the mimotopes for MAbs B6.1 and C3.1. In Fig. 1, the amount of peptide mimotope required reducing the binding of MAb B6.1 (Fig. 1A), C3.1 (Fig. 1B) to its original glycan epitope by 50% (IC50) was calculated, and peptides were compared on this basis. Based on IC_{50}, the binding affinity ranking of candidate mimotopes in descending order is PS31, PS2, PS28, PS55 and PS76.

3.3. Vaccination with three synthetic mimotope peptides induced antibody production as well as protection against disseminated candidiasis in mice

Immunizations of mimotopes in mice were done by use of the same antigen-pulsed DC based vaccine strategy favoring production of antibodies as described before [8]. Fba peptide vaccine was used as a positive control for protective efficacy. Although this vaccine approach would not be generally applicable to humans, it was used here for the rapid throughput evaluation of candidate mimotope peptides. After the first booster immunization, all five mimotopes were themselves immunogenic as shown by good antibody responses to each mimotope (Fig. 2A). Antibody ELISA titers in immune sera are highest against three mimotopes, PS31, PS2, PS28. Serum samples were also tested for their ability to bind to the original protective glycan epitope β-$(Man)_3$ by ELISA, and each mimotope was able to induce antibodies responding to original glycan β-$(Man)_3$. Consistently, the antibodies from immune sera of mice vaccinated with PS31, PS2, PS28 show higher titers of binding to the original β-$(Man)_3$ as compared to the antibody responses in anti-PS55 and anti-PS76 immune sera (Fig. 2B). Strikingly, the same three mimotopes (PS31, PS2, PS28), which elicited the best antibody responses, induced a high degree of protection as evidenced by 60–80% survival in mice challenged with the lethal dose

Table 1
The results of extensive phage library screening. Screening of phage clones present in MAb B6.1-selection pools from the phage display peptide libraries resulted in the identification of several peptide candidates that are recognized by MAbs B6.1.

Clone	Frequency[1]	Nonapeptide sequences (N → C)
PS76	7.4	YRQFVTGFW
PS31	70.4	HYKTYGGYW
PS2	14.8	WVPPGSWYL
PS28	3.7	SYLTTGGFW
PS55	3.7	SWYEGLRLI

[1] Percent of 54 total B6.1 positive phage clones selected from plaque lifts.

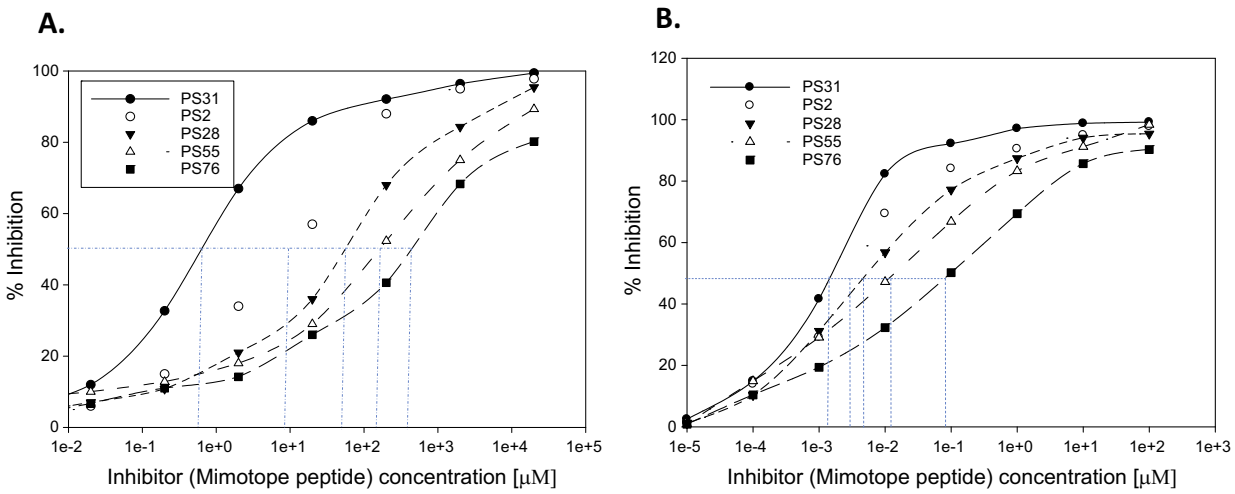

Fig. 1. ELISA inhibition data for MAbs B6.1 and C3.1. The candidate peptide mimotopes (PS31, PS2, PS28, PS55 and PS76) were tested and compared in an ELISA inhibition assay. (A) MAb B6.1 (0.025 μg/ml) was pre-incubated with various concentrations of each peptide mimotope, then the resulting solutions were added to pre-blocked β-$(Man)_3$-BSA coated microtiter plate in triplicate. (B) By a similar approach, MAb C3.1 (0.05 μg/ml) was pre-incubated with various concentrations of each peptide mimotope, then the resulting solutions were added to pre-blocked β-$(Man)_3$-BSA coated microtiter plates in triplicate. The percent inhibition of binding activity was calculated relative to wells containing antibody without mimotope inhibitor. The IC50 of each mimotope was determined as the mimotope concentration that inhibits 50% of binding activity to either MAb.

of fungus (Fig. 2C). This conclusion was further strengthened by an extended observation in which neither of the two other mimotopes PS55 or PS76 induced protection against disseminated candidiasis. Importantly, the survivors in groups immunized against PS31, PS2 and PS28 had greatly reduced or even non-detectable viable fungal units (colony forming units, CFU) in the kidney, a target organ in disseminated candidiasis (Fig. 2D), as compared to animals that succumbed ($p < 0.001$). To answer whether induced anti-mimotope antibody responses are responsible for the protection, antisera were collected from immunized mice and transferred by intraperitoneal injection (i.p.) to naïve mice 4 h before intravenously challenge with a lethal dose of *C. albicans*. Control groups were given DPBS buffer prior to the challenge. The group that received anti-Fba immune sera was used as a positive control for protection. After challenge, mice treated with the antiserum (anti-PS2, anti-PS31 and anti-PS28) had prolonged survival times as compared to the control groups (Fig. 1E), and consistently, mice that received the antiserum of the same three mimotope groups (PS31, PS2 and PS28) had significantly reduced fungal counts in their kidneys (Fig. 1F) as compared to the control group. This data provided strong evidence that anti-mimotope peptide antibodies are at least partially, if not entirely, responsible for the protection against a lethal challenge with the fungus.

3.4. Antibodies in immune sera of mice immunized with mimotopes bind to the live fungal cell surface

The serum antibodies were also tested for their binding to the cell surface of live *C. albicans* whole cells as described before [12,13]. Each of the five antisera, but not negative control sera, reacted directly with *C. albicans* yeast cells as evidenced by indirect immunofluorescence microscopy (Fig. 3A–F). MAbs B6.1 and C3.1 specific for original glycan epitope were used as positive controls, and immunofluorescence confocal microscopic analysis of *C. albicans* yeast forms showed that epitope of B6.1 and C3.1 is displayed homogeneously over the entire cell surface (Fig. 3G,H). Antibodies in immune serum samples from mice immunized with PS31, PS2 and PS28 showed similar uniform binding to yeast cell surface, confirming they are specific for the same glycan epitope abundantly expressed on the cell surface of *C. albicans* (Fig. 3B–D). However, binding of antibodies from anti-PS55 and anti-PS76 immune sera to *Candida* yeast cells was found to be patchy, with punctate staining over the cell surface (Fig. 3E,F).

3.5. Three mimotope-Fba conjugates were immunogenic in BALB/c mice

Each mimotope (PS31, PS2 and PS28) was further conjugated to the Fba peptide through GGGGS linker to form new mimotope-Fba double epitope vaccines. The antigen-pulsed dendritic cell (DC)-based vaccine strategy was used in all immunizations for development of antibodies in mice. The dendritic cell (DC)-based immunization approach was used only as a throughput evaluation of candidate short peptides. Serum samples from immunized mice were tested after the first booster for their ability to bind to both the mimotope and Fba peptide as well as the original protective glycan epitope β-$(Man)_3$ by ELISA. All three conjugates, PS31-Fba, PS2-Fba and PS28-Fba, were immunogenic as shown by high titers of specific antibody responses to each of the test antigens (Fig. 4). ELISA results demonstrate that serum antibodies bind specifically to mimotopes PS31, PS2, and PS28 (Fig. 4A), Fba peptide epitope (Fig. 4B), as well as original glycan epitope β-$(Man)_3$ trimannose (Fig. 4C).

3.6. Vaccination with mimotope-Fba conjugate vaccine induced protective immunity comparable to glycopeptide conjugate β-$(Man)_3$-Fba against disseminated candidiasis

Three mimotope-Fba conjugate vaccines (PS31-Fba, PS2-Fba, and PS28-Fba) were tested for protective efficacy induced in the BALB/c mouse model of human disseminated candidiasis due to *C. albicans*. Original β-$(Man)_3$-Fba conjugate vaccine was used as a positive control for induced protection and as a standard in protection assays to which mimotope-based vaccine constructs were compared. The immunized group that received the PS31-Fba vaccine had 80% survival throughout the 80-days post-challenge. Mice that received either PS2-Fba or PS28-Fba showed 40% and 60% survival. On the other hand, control groups given DPBS or DCs alone died within 2 weeks (Fig. 5A). Among the three mimotope-Fba conjugates, PS31-Fba conjugate induced a high degree of protection comparable to original glycopeptide β-$(Man)_3$-Fba, as evidenced

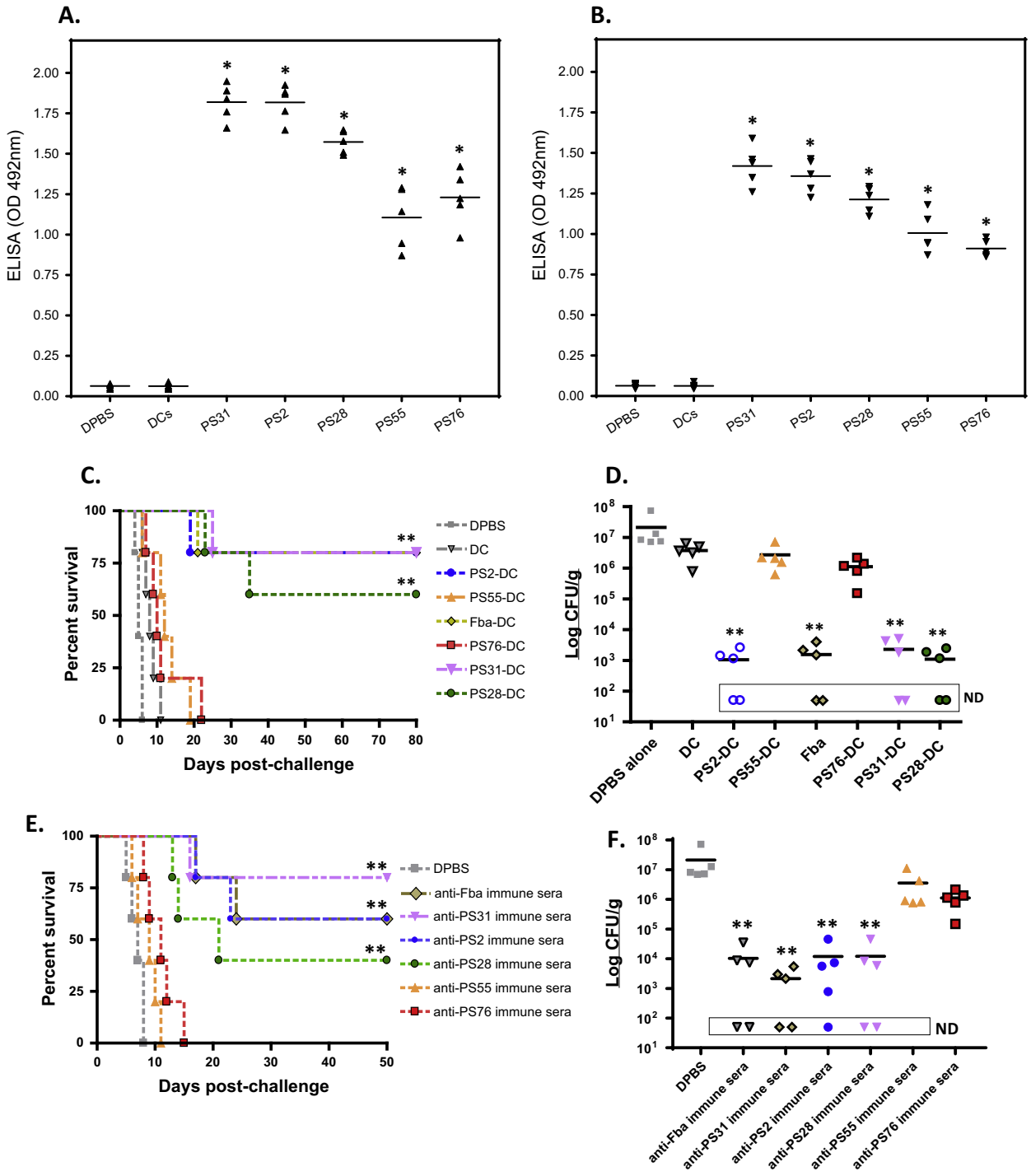

Fig. 2. Vaccination with three synthetic mimotope peptides induced antibody production and protection against disseminated candidiasis in mice. Sera from mice immunized with five synthetic mimotopes presented on dendritic cells (DCs) were tested by ELISA for antibody responses against the mimotopes (A) and the original glycan epitope (B). Control groups consisted of mice given DCs or DPBS only as indicated Serum samples were diluted 1:200 and tested by ELISA. The antibodies from immune sera of mice vaccinated with PS31, PS2, and PS28 show higher titers of binding to both the mimotopes and the original β-$(Man)_3$ as compared to the antibody responses in anti-PS55 and anti-PS76 immune sera. (C) Following challenge with a lethal dose of live *C. albicans*, mice vaccinated with PS31, PS2 and PS28 survived significantly longer than either of the control groups ($P < 0.01$). (D) Mice immunized with PS31, PS2 and PS28 had greatly reduced or non-detectable (ND) viable fungal colony forming units (CFUs) per kidney pairs as compared to control groups ($p < 0.001$). For each group, each data point only represents detectable CFU per mouse; non-detectable CFUs (<50 per kidney pair) were shown as lower line labeled "ND". (E) Sera were collected from immunized mice and were pooled and tested for passive protection of naive mice against experimental disseminated candidiasis. Enhanced protection against disseminated *C. albicans* infection was observed in mice that received sera from mice immunized with PS31, PS2 and PS28 mimotope as compared to animals that received control materials ($p < 0.01$). (F) Mice received immune sera from groups of PS31, PS2 and PS28 had significantly reduced or non-detectable (ND) viable CFUs per kidney pairs as compared to DPBS control and either of PS55 and PS76 groups ($p < 0.001$). Statistical analysis: $^{*}p < 0.05$ as statistically significant, $^{**}p < 0.01$, and $p < 0.001$ indicated in caption as statistically highly significant.

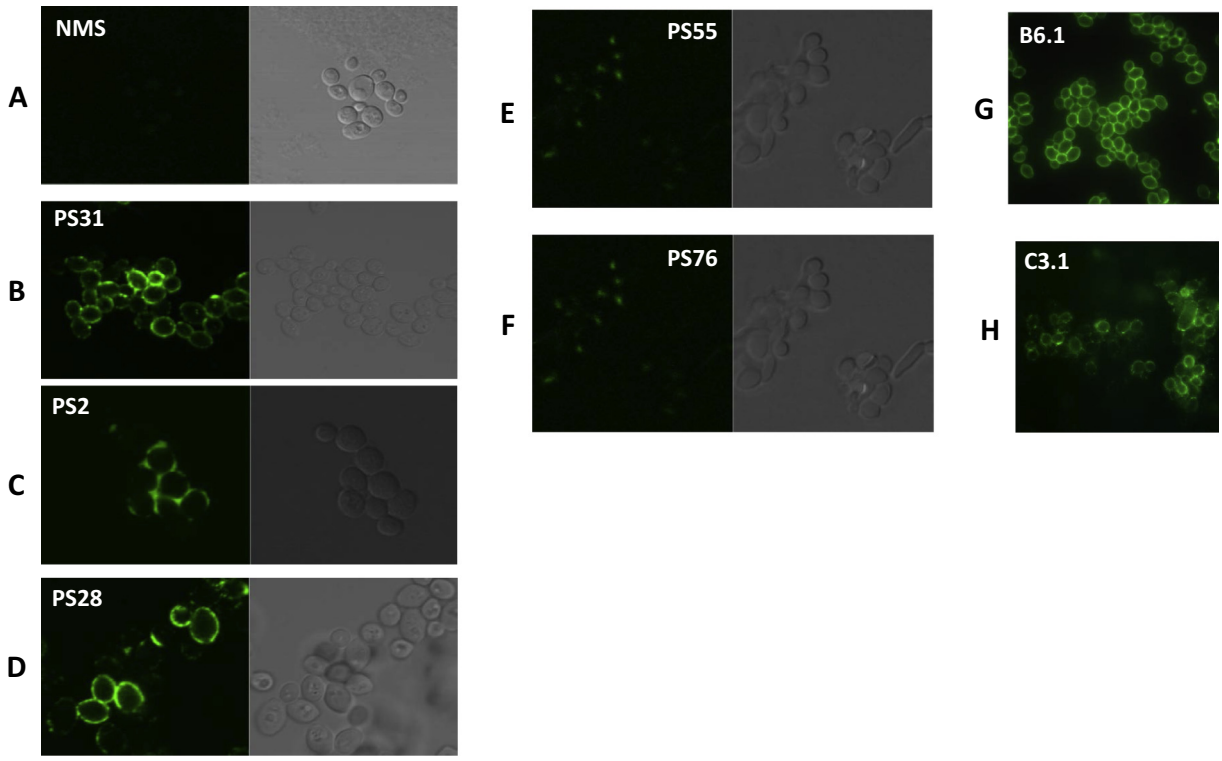

Fig. 3. Immune serum from mimotope vaccinated mice recognized epitopes on the surface of *C. albicans* yeast cells. The serum antibodies were tested for their binding to cell surface of live *C. albicans* 3153A yeast cells. Each of the five antisera (B-F), but not negative control sera (normal mouse serum, NMS) from DPBS group (A), reacted with *C. albicans* yeast cells as evidenced by indirect immunofluorescence microscopy. The epitope displayed by anti-PS31, PS2 and PS28 immune sera (B–D) was similar to MAbs B6.1 (G) and C3.1 (H), which are specific for the original glycan epitope and were used as positive controls. However, epitope binding of antibodies from anti-PS55 and anti-PS76 immune sera to *Candida* yeast cells was found to be patchy, with punctate staining over the cell surface (E, F). Left panels with a dark background have laser illumination (488 nm); right panels are illuminated with white light.

by prolonged survival, and significantly reduced kidney fungal burden in mice challenged with the fungus (Fig. 5B). PS2-Fba and PS28-Fba groups also had reduced kidney burden in mice with disseminated candidiasis (Fig. 5B).

To confirm that induced antibodies were protective, sera from mice immunized against each mimotope-Fba conjugate were transferred to naïve mice as described before, and 4 h later challenged with a lethal dose of live *C. albicans* yeast cells. The mice treated with immune sera had significantly prolonged survival times as compared to negative controls that received either DPBS buffer alone or normal mouse sera from naïve mice (Fig. 5C). Consistently, immune sera from the PS31-Fba group provided comparable protection in naïve mice as anti-β-$(Man)_3$-Fba immune sera. Immune sera from either PS2-Fb or PS28-Fba group also provided some protection in mouse recipients. Mice receiving treatment of immune sera from vaccinated mice also had significantly reduced or non-detectable viable fungal CFU in kidney as compared to the DPBS group or mice receiving normal mouse sera. Mice receiving either anti-PS-31 or anti-β-$(Man)_3$-Fba immune sera had the least fungal burden in the kidney as compared to other groups treated with immune sera (Fig. 5D).

4. Discussion

In this study, the mimotope concept is applied to the field of epitope-specific vaccination against disseminated candidiasis. Although we initially demonstrated a glycopeptide-TT vaccine as an effective formula feasible for human use, the synthesis of the glycan part and subsequent chemical conjugation is difficult, expensive, and requires much expertise, ultimately precluding any further development of glycopeptide vaccines. Critically, drawbacks of carbohydrate-based vaccines generally include difficulties in glycan analysis, purification, synthesis, and the low affinity of anti-carbohydrate antibodies as compared to anti-peptide or anti-protein antibodies [30,31]. Experimental observations and theoretical calculations indicate that the decrease in entropy that accompanies most sugar/protein interactions explains the low affinity. The poor quality of antibody responses to carbohydrates is a major obstacle associated with developing carbohydrate-based vaccines [30]. Molecular mimics (mimotopes) of immunogenic epitopes of carbohydrate antigens represent an attractive alternative that can avoid these problems yet induce protective immunity. They can also be produced relatively easily and cheaply, with the potential for production as polyvalent formulations. Therefore, peptide mimics of carbohydrate structures have enormous potential as safe and cheap vaccine components.

The overall goal of this research is to preserve the protective duality of a glycopeptide β-$(Man)_3$-Fba vaccine, retain the range of the induced immunity against *Candida* species of medical significance, and simplify existing glycopeptide vaccine constructs for production. The glycan part of the glycopeptide vaccine, β-$(Man)_3$ is apparently expressed by all *C. albicans* isolates, and by several *Candida* species of medical significance. A panel of novel peptide mimotopes that structurally mimic this protective glycan epitope β-$(Man)_3$ were used as surrogate immunogens that substitute for the glycan part of glycopeptide vaccine. Use of antibody affinity chromatography and a phage display peptide library (PDPL) have defined a family of peptides that are recognized by MAb B6.1. Each of these peptides is nine amino acids in length (nonapeptides), and appears to mimic a carbohydrate epitope, as evidenced by reactivity with MAb B6.1. Five phage clones presenting the best results of specificity and selectivity were selected and evaluated in competi-

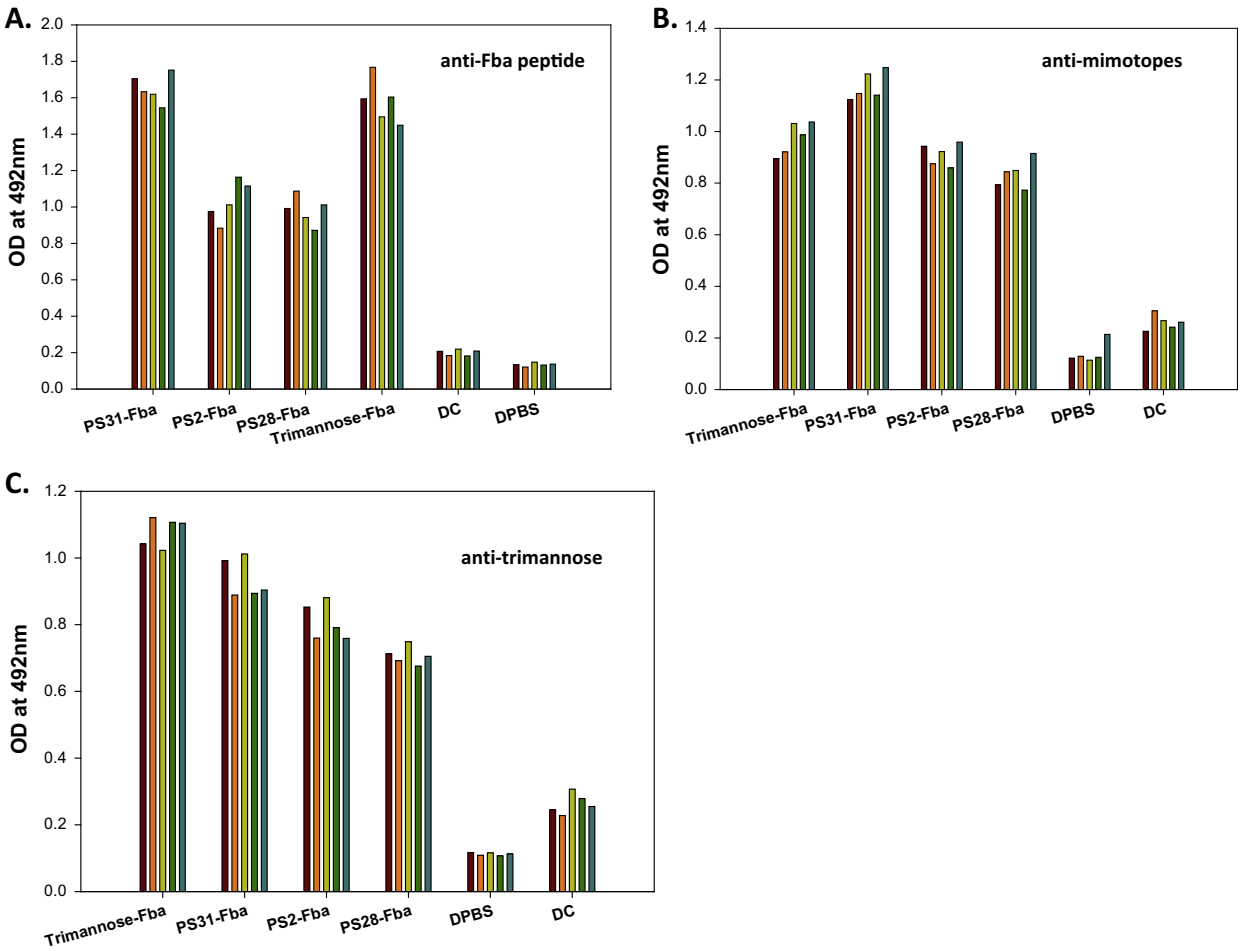

Fig. 4. Antibody responses in mice against the mimotope-Fba vaccines. Sera from mice immunized with three mimotope-Fba conjugates were tested by ELISA for antibodies against the Fba peptide (A), synthetic mimotopes (B), and synthetic trimannose [β-$(Man)_3$] (C). Serum samples were collected from vaccinated mice 14 days after the first booster, diluted 1:100 and tested by ELISA. Sera from groups of mice injected with DPBS or DCs were used as negative controls. Serum from mice vaccinated with β-$(Man)_3$-Fba was used as positive control.

tion ELISA and then vaccination experiments in BALB/c mice. The IgM-binding peptides may represent potential artifacts when PDPL are used to epitope map IgM MAbs, because such peptides appear to bind to an IgM MAb in a manner analogous to antigen. Therefore, the specificity of binding and antigen inhibition are tested by competition ELISA first, by which specific binding of each mimotope to MAbs B6.1 and C3.1 was measured and confirmed. Synthetic peptide mimotopes were able to inhibit the binding of either B6.1 or C3.1 to its carbohydrate epitope and is dose-dependent. Based on ELISA data and the demonstration of dose-response inhibition in these experiments, mimotopes PS31, PS2, PS28 showed stronger ELISA reaction with MAbs B6.1 and C3.1, as compared to PS55 and PS76. Furthermore, all five mimotopes were selected and evaluated in vaccination experiments in BALB/c mice by dendritic cell (DC) based immunization approach. Although this vaccine approach would not be generally applicable to humans, it was used here only for the rapid throughput evaluation of candidate mimotope peptides. Furthermore, souse bone marrow (BM) cells generated DCs when cultured in the presence of GM-CSF and IL-4, mimicking the process of differentiation of monocytes into DCs [32,33]. Both LPS and PGE2 were used to induce DC maturation in vitro. LPS-activated DCs are thought to follow a set program in which they secrete inflammatory cytokines (such as IL-12) and then become refractory to further stimulation (i.e. "exhausted") [34,35]. However, enough evidences also show that LPS activation does not exhaust DCs but rather primes them for subsequent signals from T cells [36,37]. These data suggest that it is time to re-examine well-established views about DC differentiation. In our protocol, PGE_2 was also used as an endogenous anti-apoptotic factor for DC and promotes the survival of DC loaded with antigen in vitro and in vivo [38].

Our study shows that all five peptide mimotopes were able to induce specific antibody responses, and three out of five peptide mimotopes have been identified and demonstrated to be able to induce the same protective efficacy as β-$(Man)_3$ against disseminated candidiasis in mice. In addition, mice treated with the antiserum (anti-PS2, anti-PS31 and anti-PS28) had prolonged survival times and significantly reduced fungal counts in their kidneys as compared to the control group. Passive transfer results demonstrated that the induced anti-mimotope antibody responses were responsible for the protection; therefore, such peptide mimotopes have the potential of replacing the glycan part, which will obviate the need for complex carbohydrate synthesis in the final vaccine construct.

To investigate the protective mechanism of the mimotope vaccines, the reactivity of antibodies in antisera with glycan epitope on live fungal yeast cells was confirmed by indirect immunofluorescence microscopy. MAbs IgM B6.1 and IgG3 C3.1, which both bind to abundantly expressed cell surface epitope β-$(Man)_3$ and protect mice against disseminated candidiasis [14,15], were used

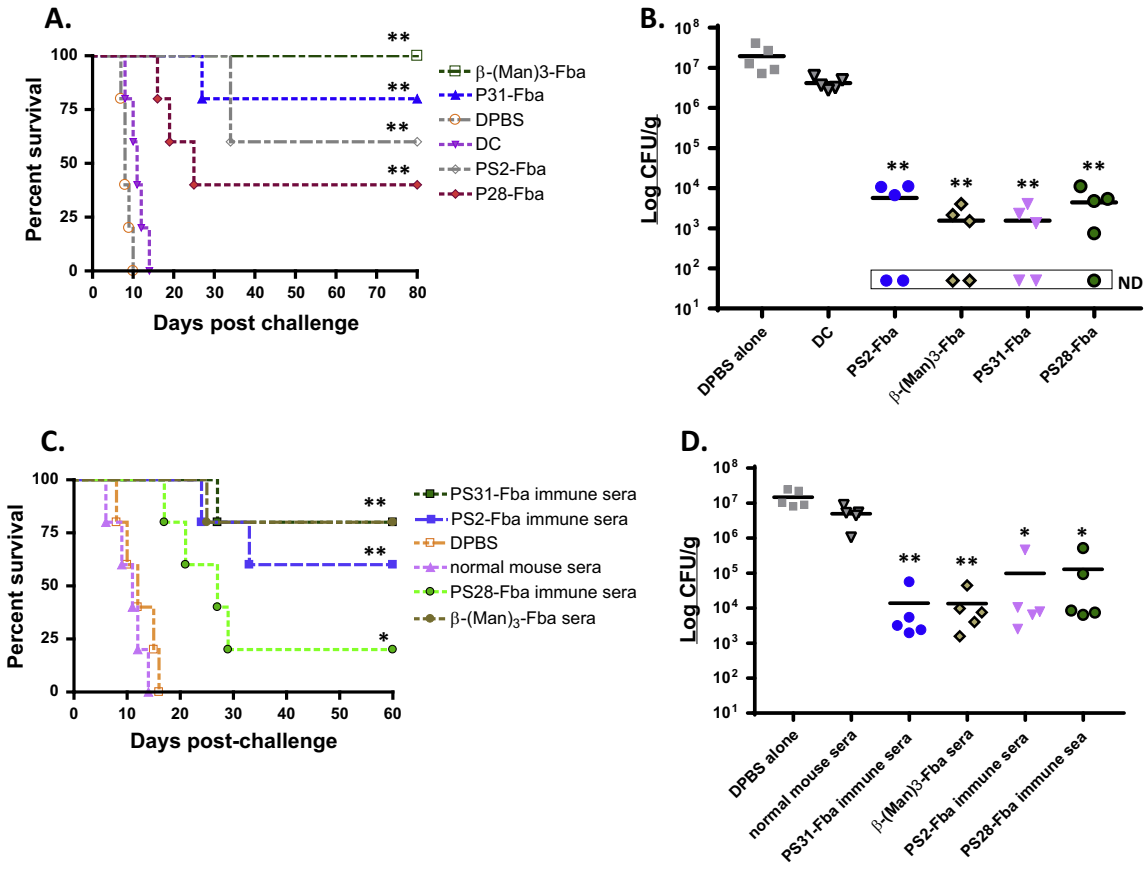

Fig. 5. Mimotope-Fba conjugate vaccines induce protective responses in mice against disseminated candidiasis. Mice vaccinated with β-$(Man)_3$-Fba conjugate was used as positive control. (A) Mice vaccinated with conjugates PS31-Fba, PS2-Fba and PS28-Fba survived significantly longer than control mice that received DPBS buffer or DCs alone ($p < 0.00$). (B) Mice immunized with PS31, PS2 and PS28 had greatly reduced or non-detectable (ND) viable fungal CFUs per kidney pairs as compared to either of the control groups ($p < 0.001$). (C) Antibody is responsible for protection against disseminated candidiasis. Serum was collected from mice vaccinated with mimotope-Fba conjugates. Immune sera were pooled and tested for passive protection of naive mice against experimental disseminated candidiasis. (D) Naïve mice received treatment of immune sera pooled from vaccinated mice had significantly reduced or ND viable fungal CFUs in kidney as compared to DPBS group or mice receive normal mouse sera. $^{*}p < 0.05$ as statistically significant, $^{**}p < 0.01$, and $p < 0.001$ indicated in caption as statistically highly significant.

as a positive control for serum IgM/IgG antibody binding to the fungal cell surface. The uniform binding pattern of B6.1 and C3.1 suggest a dense distribution of epitopes displayed on the yeast surface [23]. Strikingly, the binding pattern of antibodies in PS31, PS2 and PS28 immune sera is identical to the distribution described for the epitope recognized by the protective MAbs B6.1 and C3.1 [23], which is in contrast to the patchy or dotting distribution of epitope recognized by the non-protective anti-PS76 and anti-PS55 immune sera. Evidence supports that three key factors for antibodies to be protective are appropriate specificity, isotype and sufficient titers. Our previous studies revealed that β-$(Man)_3$ specific MAb B6.1 enhances mouse neutrophil candidacidal activity [39] and that protection by B6.1 or its murine IgG3 isotype variant requires host complement [11]. It is likely that mimotope specific antibodies in immune sera protect immunized mice against invasive candidiasis by the same mechanism as MAbs B6.1 and C3.1, since they all bind to the *C. albicans* cell surface β-$(Man)_3$ in same pattern.

Interestingly, all three mimotope-Fba conjugates were able to provide comparable dual immune recognitions and protection as the original β-$(Man)_3$-Fba conjugate, which may ensure protective immunity against mutant strains of the fungus that may have lost one of the two epitopes. To that end, identification of mimotopes against other important *C. albicans* cell wall antigens might provide even greater protection when combined with mimitope-Fba vaccines. Ultimately, combining peptide mimotopes against multiple cell wall components may enhance the efficacy of a candida vaccine, whether they are conjugated to mimotope-Fba conjugate or other carriers in combination with a potent adjuvant. Such a multivalent vaccine could representing immunodominant antigens to create an additive or synergistic immune response.

Our early work shows that MAbs specific for *C. albicans* cell surface β-1, 2-mannotriose [β-$(Man)_3$] protect against both disseminated candidiasis and vaginal infection in mouse models of disease [11,14,15,23,28]. Since the three protective mimotopes mimic the specificity of this key glycan that is apparently expressed 100% by three non-albicans *C. tropicalis, C. glabrata, C. guilliermondii* and *C. lusitaniae*, the corresponding mimotope-peptide vaccines may also have the potential to provide broad protection against the medically important *Candida* species, in addition to *C. albicans.* In future studies, we will initiate experiments to test the ability of induced immune responses to protect against disseminated candidiasis due to other *Candida* species [40,41]. We have established immunosuppressed mouse models of disseminated candidiasis by the two clinically important *NAC* species, *C. glabrata and C. tropicalis* [42], therefore, *we* will use these established mouse dissemination models for mimotope-Fba conjugate vaccines as a paradigm for the study of the other *Candida* species.

Our previous study has demonstrated that the addition of the TT to the glycopeptide conjugate provided sufficient self-adjuvanting activity to allow us to omit the DC approach as well as the need for adjuvant [10]. Following this successful formulation of glycopeptide vaccine feasible for human use, we have confi-

dence that our approaches will result in a new mimotope-peptide-TT vaccine. As well as being effective and safe for human use, the improved new mimotope-Fba (double peptide) vaccine constructs are more economical to produce. Applications are likely to include new vaccines for which polysaccharide conjugates have failed [43,44], thus furthering mass vaccination programs for resource-limited developing countries where the burden of infectious disease is greatest [45].

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vaccine.2019.03.061.

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