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6-*O*-Branched Oligo-β-glucan-Based Antifungal Glycoconjugate Vaccines

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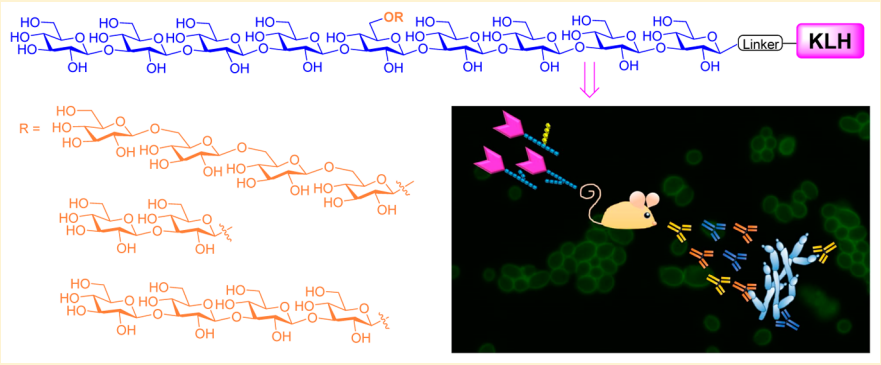
Supporting Information



ABSTRACT: With the rapid growth in fungal infections and drug-resistant fungal strains, antifungal vaccines have become an especially attractive strategy to tackle this important health problem. β-Glucans, a class of extracellular carbohydrate antigens abundantly and consistently expressed on fungal cell surfaces, are intriguing epitopes for antifungal vaccine development. β-Glucans have a conserved β-1,3-glucan backbone with sporadic β-1,3- or β-1,6-linked short glucans as branches at the 6-*O*-positions, and the branches may play a critical role in their immunologic functions. To study the immunologic properties of branched β-glucans and develop β-glucan-based antifungal vaccines, three branched β-glucan oligosaccharides with 6-*O*-linked β-1,6-tetraglucose, β-1,3-diglucose, and β-1,3-tetraglucose branches on a β-1,3-nonaglucan backbone, which mimic the structural epitopes of natural β-glucans, were synthesized and coupled with keyhole limpet hemocyanin (KLH) to form novel synthetic conjugate vaccines. These glycoconjugates were proved to elicit strong IgG antibody responses in mice. It was also discovered that the number, size, and structure of branches linked to the β-glucan backbone had a significant impact on the immunologic property. Moreover, antibodies induced by the synthetic oligosaccharide−KLH conjugates were able to recognize and bind to natural β-glucans and fungal cells. Most importantly, these conjugates elicited effective protection against systemic *Candida albicans* infection in mice. Thus, branched oligo-β-glucans were identified as functional epitopes for antifungal vaccine design and the corresponding protein conjugates as promising antifungal vaccine candidates.

KEYWORDS: *carbohydrate, β-glucan, glycoconjugate, vaccine, fungus*

Fungal infections, mostly caused by *Candida*, have drastically increased in the past decades,[1−3] which poses a serious health problem. On the other hand, the efficacies of antifungal drugs have been impeded by their severe side effects and the emergence of drug-resistant fungal strains. As a result, deep-seated fungal infections in nosocomial settings have high mortalities,[2,4] and alternative therapeutic strategies, such as antifungal vaccines, are desirable.[5,6]

For antifungal vaccine development, polysaccharides,[7] such as β-glucans,[8,9] in the cell wall glycocalyx of pathogenic fungi are attractive targets,[10] as they are exposed on cells and can elicit strong immune response.[8,9] β-Glucans[7,11] are a class of polysaccharides composed of ca. 1500 β-1,3-linked D-glucose units with 40−50 additional β-1,6- or β-1,3-linked glucans as branches at the main-chain glucose 6-*O*-positions.[12,13] β-Glucans are highly conserved on all pathogenic fungal cells[11] because they play important functional roles, such as keeping the mechanical strength and integrity of fungal cells.[14] Moreover, β-glucans are dectin-1 ligands, which can help the uptake of their conjugates by dendritic cells, thereby enhancing their immunogenicity.[15] Thus, β-glucans have incited great interest in antifungal vaccine development. For instance, the protein conjugate of a natural β-glucan was found to elicit effective protection against *Candida albicans* and *Aspergillus fumigatus* infections in a mouse model.[16]

In the past two decades, antibacterial glycoconjugate vaccines have made great progresses,[5−9] but there is still no clinically

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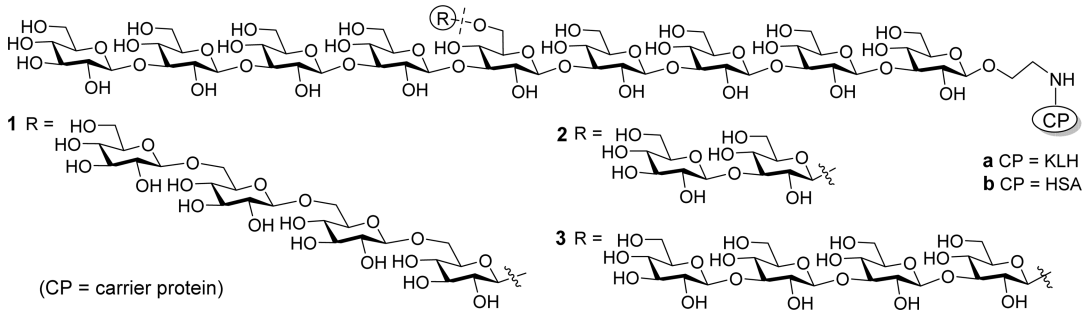
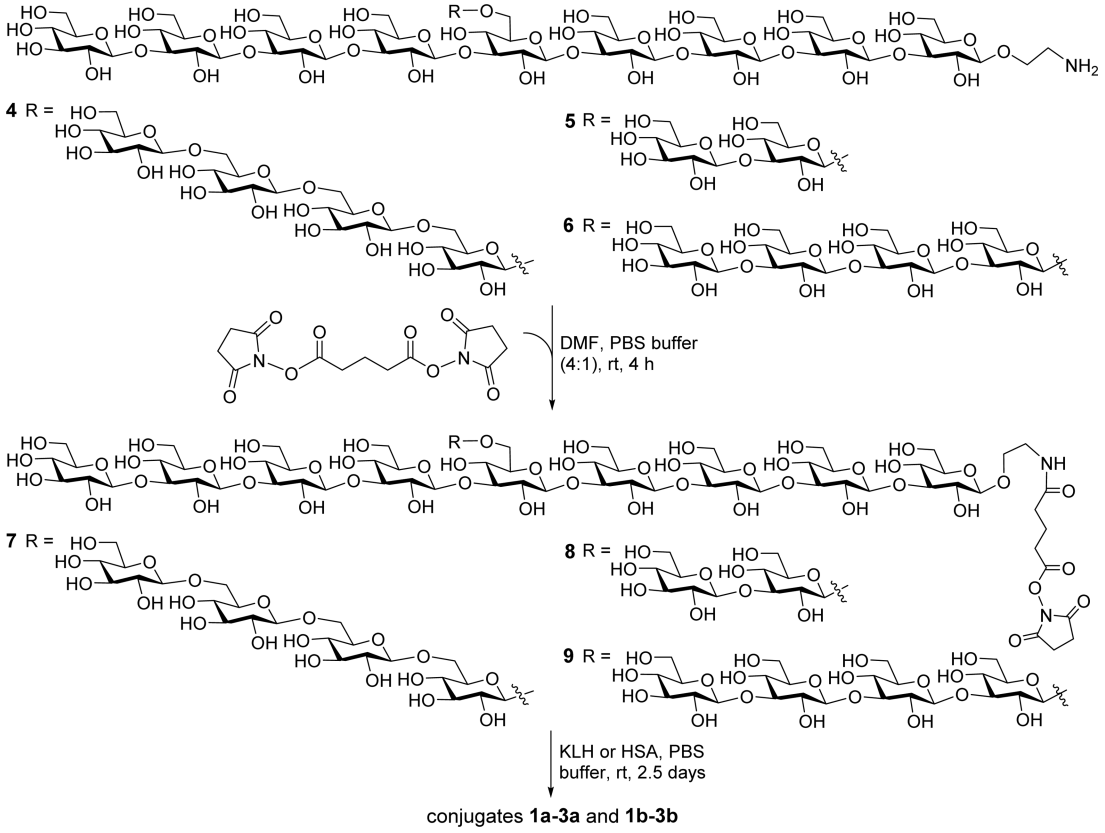


Figure 1. Designed protein conjugates **1**–**3** of β-glucan oligosaccharides with β-1,6-tetraglucose (**1**), β-1,3-diglucose (**2**), and β-1,3-tetraglucose (**3**) branches at the 6-*O*-position of the central glucose unit of a nonasaccharide.

Scheme 1. Preparation of the β-Glucan Oligosaccharide–Protein Conjugates



useful antifungal glycoconjugate vaccine. One of the reasons is that the correlations between the structures of fungal carbohydrate antigens, such as β-glucan, and their antigenicity are unclear. To decipher the structure–activity relationships of β-glucans for the design and optimization of antifungal vaccines, glycoconjugates composed of structurally defined and homogeneous synthetic oligosaccharides should be very useful. Such vaccines, compared to vaccines composed of natural polysaccharides and as demonstrated in antibacterial vaccines,[17−22] also have other advantages, such as improved purity and reproducibility, easier quality control, and absence of microbial contaminants.

Recently, several linear and a branched oligo-β-glucans were synthesized and studied. Their protein conjugates could elicit immune responses[23−26] comparable to that elicited by the CRM_{197} conjugate of laminarin (Lam), a natural β-glucan with sporadic 6-*O*-branches.[16] However, it was found that only linear oligo-β-glucans,[23,25] not the branched one,[23] elicited protective immunities against fungal infections in mouse. These results were in contrast to that of the Lam conjugate.[16] Clearly, the immunologic role of β-glucan branches is undefined.

RESULTS AND DISCUSSION

To better understand β-glucans and uncover the proper oligosaccharide epitopes for vaccine design, we prepared the keyhole limpet hemocyanin (KLH) conjugates **1a**–**3a** (Figure 1) of three branched oligo-β-glucans with different β-1,6-tetraglucose (**1a**), β-1,3-diglucose (**2a**), and β-1,3-tetraglucose (**3a**) branches at the central sugar unit 6-*O*-position of the same β-1,3-nonaglucan backbone. They were designed to simulate and cover the immunologic determinant epitopes of natural β-glucans,[19] such as that with short or long β-1,3-glucan and β-1,6-glucan branches. These glycoconjugates were tested in mice to study their immunologic properties and their capabilities to elicit protection against fungal infections. Herein, KLH was used as the carrier protein due to its easy accessibility and wide

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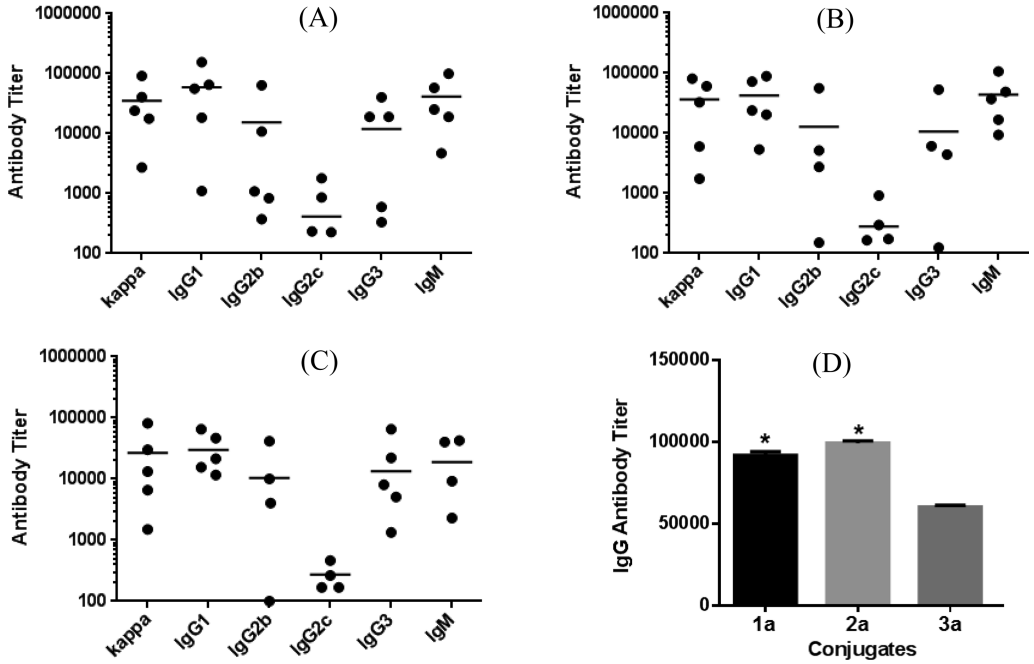

Figure 2. ELISA results of the day 48 antisera of individual mice inoculated with conjugates **1a** (A), **2a** (B), and **3a** (C), with the antibody titer of each mouse shown as a dot and the group average as a black bar, as well as the total IgG antibody titers of the pooled day 48 antisera of each group of mice inoculated with **1a**–**3a** (D). (∗) Titer difference from that of **3a** is statistically significant ($P < 0.05$).

applications,[27] although clinically it is not necessarily the ideal carrier protein for antifungal vaccines. Nevertheless, it is suitable for immunologic and structure–immunogenicity relationship studies of the designed oligosaccharides and for revealing the proper antigens for vaccine development. They would also facilitate the comparison of **1a**–**3a** with the reported KLH conjugates of linear oligo-β-glucans[28] to gain more insights into structure–activity relationships of oligo-β-glucans. Moreover, the human serum albumin (HSA) conjugates **1b**–**3b** (Figure 1) of the oligo-β-glucans were prepared, which were used as coating antigens for enzyme-linked immunosorbent assays (ELISA) of carbohydrate antigen-specific antibodies.

Preparation of Glycoconjugates. Oligosaccharides **4**–**6**, which were prepared according to a reported procedure,[29] were employed to couple with KLH and HSA through a bifunctional glutaryl linker (Scheme 1). This simple linker was selected because our previous studies proved that its conjugation reactions using activated glutaryl esters were easy and effective and it would not elicit antibodies against itself or affect the immunologic property of resultant glycoconjugates.[30−32] Consequently, **4**–**6** were treated with excessive disuccinimidyl glutarate (DSG, 15 equiv) in DMF to form activated monoesters **7**–**9**, which were readily purified via a precipitation process. Reaction of **7**–**9** with KLH and HSA in PBS afforded **1a**,**b**–**3a**,**b**, which were purified with a Biogel A0.5 column. We have demonstrated that active esters of *N*-hydroxysuccinimide were stable to PBS under natural conditions.[32] The glucose contents of conjugates **1a**,**b**–**3a**,**b** were assessed by using the phenol–sulfuric acid method[33] with KLH and HSA as blanks to exclude any potential interference caused by the glycans present in the proteins. The carbohydrate loadings of HSA conjugates **1b**–**3b** were also assessed with MS (Supporting Information), on the basis of the difference in molecular weights of a specific conjugate and its corresponding carrier protein. The two methods afforded similar results of carbohydrate loading for **1b**, **2b**, and **3b**, that is, 12.1, 15.2, and 15.5% with MS and 9.8, 15.1, and 13.7% with the phenol–sulfuric acid method, respectively. Again, this work confirmed that the results of sugar analysis obtained with the phenol–sulfuric acid method were reliable,[32] and the method could be utilized to assess the carbohydrate loadings of the KLH conjugates **1a**–**3a**, the molecular weights of which were too big for MS analysis. The conjugation of oligo-β-glucans with KLH was further verified with SDS-PAGE, which showed the increase in molecular mass of **1a**–**3a** as compared to KLH. The results have demonstrated that conjugation reactions between **7**–**9** and proteins were efficient, and the antigen loading levels of **1a**,**b**–**3a**,**b** were in the desirable range.[34]

Immunologic Study of Glycoconjugates. The immunologic properties of **1a**–**3a** as vaccines were evaluated in female C57BL/6J mice. Each conjugate was mixed with Freund's complete adjuvant (CFA) to form an emulsion that was injected intramuscularly (im) to each group of five mice. After the initial immunization, mice were boosted four times on days 14, 21, 28, and 38 via subcutaneous (sc) injection of the same emulsion. Blood samples were collected on day 0 before the initial immunization (blank control) and on days 21, 27, 38, and 48, respectively. Antisera were prepared from clotted blood samples according standard protocols and analyzed by ELISA with the corresponding HSA conjugates **1b**–**3b** as capture reagents to detect the oligosaccharide-specific antibodies while avoiding the interference of anti-KLH antibodies.

ELISA results (Figure 2) suggested that all three conjugates **1a**–**3a** provoked high titers of antigen-specific total (anti-kappa) antibodies (Figure 2A–C). We further demonstrated by ELISA that the antisera obtained from mice immunized with KLH and CFA did not show any binding to capture reagents **1b**–**3b**. Analysis of individual antibody isotypes revealed the production of high levels of IgM, IgG1, IgG2b, and IgG3 antibodies (Figure 2A–C), as well as a low level of IgG2c antibody. The induction of strong IgG, especially IgG1 and IgG2b, antibody responses indicated T cell-dependent

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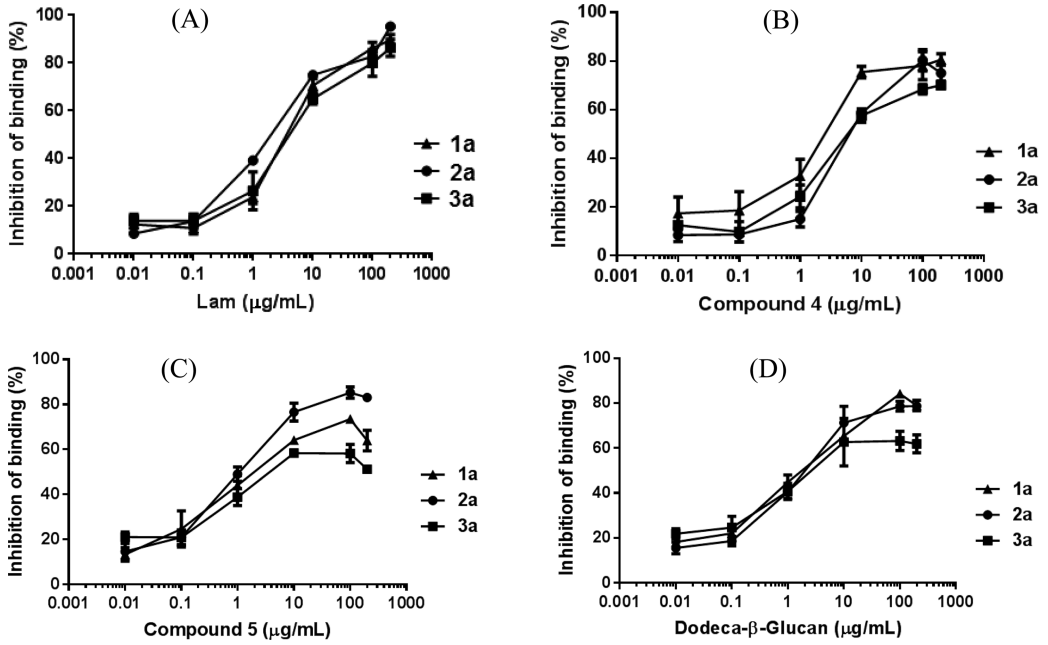

Figure 3. Competitive inhibition of the binding between conjugates **1b**−**3b** and the pooled antisera obtained with **1a**−**3a** by Lam (A), **4** with a 1,6-linked branch (B), **5** with a 1,3-linked branch (C), and linear dodeca-β-glucan (D). The error bar shows the standard error of means of three parallel experiments.

immunity, because "to achieve class switch to IgG antibodies, the B cells need to interact with helper T cells."[18] Moreover, it was also reported that IgG1 and IgG2b antibodies had higher antigen binding affinity than other antibodies,[35,36] so these two isotypes of antibodies are considered protective[37,38] and should be functional at mediating the microorganism killing, although the mechanisms of action of anti-β-glucan antibodies are not completely understood.[39] Nonetheless, the immunologic properties of **1a**−**3a** as prophylactic vaccines are desirable.

It was also observed that **1a** and **2a**, which had antigens with a β-1,6-linked tetraglucose and β-1,3-linked diglucose branches, elicited similar titers of total IgG antibodies, 91866 and 99196 respectively, that were higher than the total IgG antibody titer of **3a** (60219) with a β-1,3-linked tetraglucose branch (Figure 3D). These results suggested that **1a** and **2a** were more immunogenic than **3a**. Nonetheless, **3a** induced robust and consistent immune responses in all tested mice.

Binding Assays of the Antisera to Various β-Glucans and Fungal Cells. To probe whether antibodies elicited by **1a**−**3a** could recognize natural β-glucans, we analyzed the influence of Lam, a β-glucan carrying sporadic branches at the main-chain 6-*O*-positions, on the binding between synthetic oligo-β-glucans and anti-**1a**−**3a** sera. Antisera (1:900 dilution) were mixed with various concentrations (0, 0.01, 0.1, 1, 10, 100, and 200 μg/mL) of Lam and then applied to ELISA with HSA conjugates **1b**−**3b** as capture antigens. Antibody binding to Lam was shown by the decrease in the amount of antibodies bound to **1b**−**3b** on the plates due to Lam-caused competitive binding inhibition, which was calculated according to the equation presented under Methods. Our results (Figure 3A) showed that Lam inhibited antibody binding to **1b**−**3b** in a concentration-dependent manner with 50% inhibition concentrations (IC_{50}) of 4.3, 2.2, and 7.8 μg/mL for **1a**, **2a**, and **3a**, respectively, and at the 200 μg/mL concentration, the inhibition was >90% in all cases. Clearly, antibodies elicited by **1a**−**3a** could recognize the structural epitopes of natural β-glucan, whereas Lam showed the strongest inhibition on the binding between the antiserum of **2a** and the corresponding oligosaccharide in **2b**.

We have also evaluated the influence of free oligosaccharides, including branched β-glucans **4** and **5** and linear dodeca-β-glucan, on the binding between antisera of **1a**−**3a** and corresponding oligo-β-glucan antigens. It was shown that **4** had slightly stronger inhibition (IC_{50} = 1.9 μg/mL) on the binding between **1b** and antiserum **1a** than on the binding between **2b** and antiserum **2a** (IC_{50} = 8.0 μg/mL) or between **3b** and antiserum **3a** (IC_{50} = 6.5 μg/mL) (Figure 3B). On the other hand, **5** had a slightly stronger inhibition (IC_{50} = 1.1 μg/mL) on the binding between **2b** and antiserum **2a** than on the binding between **1b** and antiserum **1a** (IC_{50} = 2.0 μg/mL) or between **3b** and antiserum **3a** (IC_{50} = 3.7 μg/mL) (Figure 3C). These results suggested that **1a**−**3a** did elicit some antibodies specific to the unique structural motif of their oligosaccharide antigens, which are a β-1,6-linked glucan branch in **1a** and a short disaccharide branch in **2a**, but the majority of antibodies in the antisera were against a common motif. It was further disclosed that linear dodeca-β-glucan had a similar and strong inhibition on the binding of all three antisera to respective antigens (IC_{50} = 1.9, 1.8, and 2.4 μg/mL for **1a**, **2a**, and **3a**, respectively; Figure 3D). The results of these preliminary studies led us to propose that the common structural motif recognized by the majority of antibodies elicited by **1a**−**3a** was probably the main-chain structure of β-glucans. This is a very interesting observation worthy of further detailed investigations.

The binding of antisera with *C. albicans* (HKCA) cell was studied by immunofluorescence (IF) assay. A hHeat-killed HKCA cell was treated first with BSA blocking buffer to mask potentially nonspecific protein binding sites on the cell surface and then incubated with pooled antisera obtained with **1a**−**3a**. Finally, the cell was stained with fluorescein isothiocyanate (FITC)-labeled goat anti-mouse kappa antibody and examined

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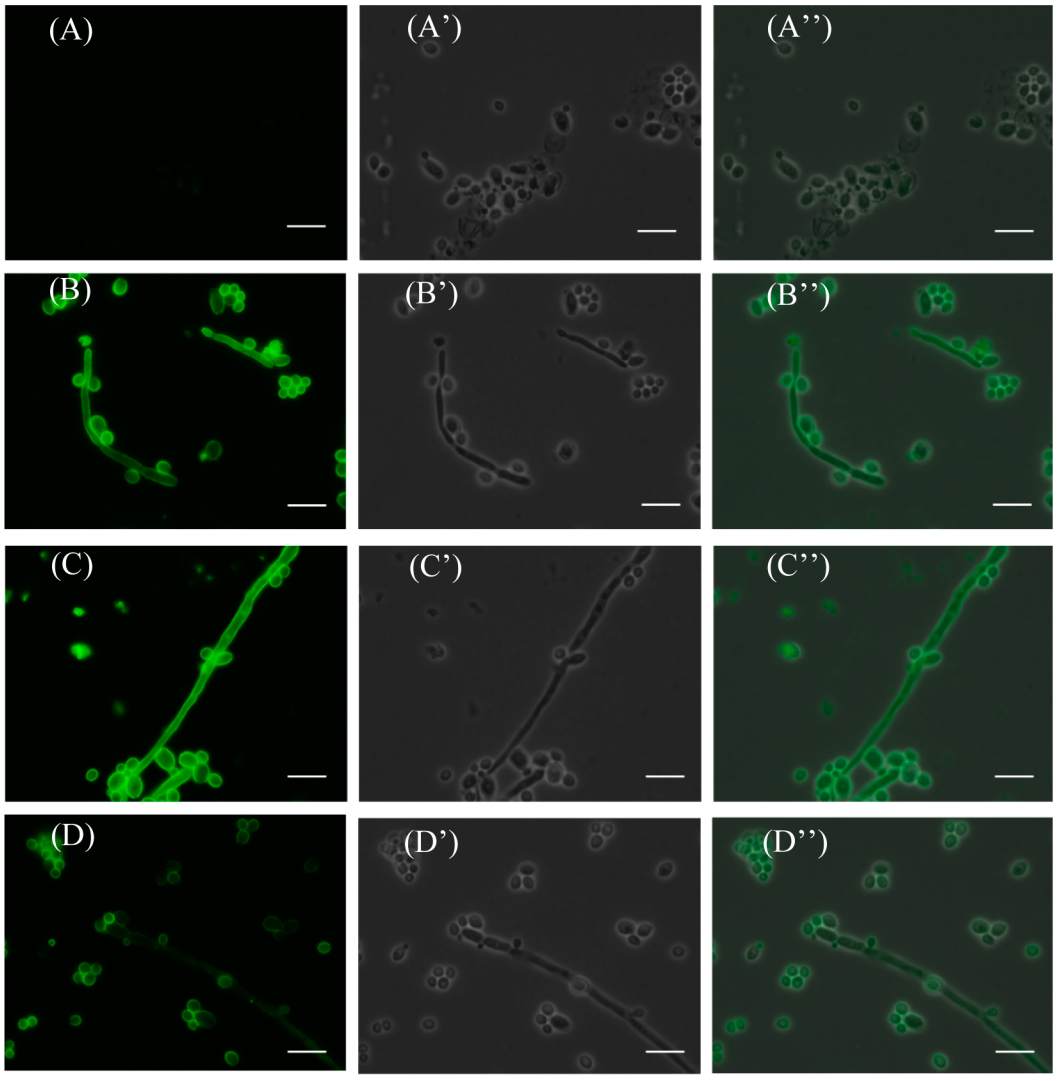

Figure 4. IF staining results of the heat-killed *C. albicans* cells using sera obtained from mice (A) without vaccine treatment (negative control) or immunized with glycoconjugates (B) **1a**, (C) **2a**, and (D) **3a**. Associated panels A′−D′ show the corresponding bright field images, and associated panels A″−D″ show the merged images. The length of the white bar is 10 μm.

with a microscope. The results (depicted in Figure 4) showed that compared to the negative control (panel A), both the fungal particles and hyphal cells were uniformly IF-stained, indicating the strong binding of antisera to the HKCA cell.

Protection against Fungal Infection. To validate the new conjugates as antifungal vaccines, **1a** and **3a**, the carbohydrate antigens of which had the same length of side chains but different glycosyl linkages, were evaluated for their abilities to protect animals from fungal infection using a mouse model.[16,23] The fungal cell used was *Candida albicans* (strain SC5314), one of the most common pathogenic fungi in clinic.[40] Each group of 11 mice was immunized four times with **1a**, **3a**, or PBS buffer (negative control). Meanwhile, literature studies revealed that, similar to PBS, CFA alone did not elicit protection against *C. albicans*,[16,23] and as mentioned above, KLH did not induce specific antibodies against *C. albicans*, which could also be considered as the negative controls for this study. Here, only **1a** and **3a** were selected for in vivo studies because their carbohydrate chains had the same length but different glycosidic linkages. After positive immune responses to **1a** and **3a** were confirmed, a predetermined lethal dose of *C. albicans* cells (7.5×10^5 cells/mouse in 200 μL of PBS) was iv injected into each mouse. The mice were monitored, and their survival time and rate are shown in Figure 5.

As depicted in Figure 5, mice in the control group started to die on day 5 after *C. albicans* challenge, and all died of fungal infection in 10 days. No deaths occurred in the mice immunized with **1a** and **3a** until days 8 and 7, respectively,

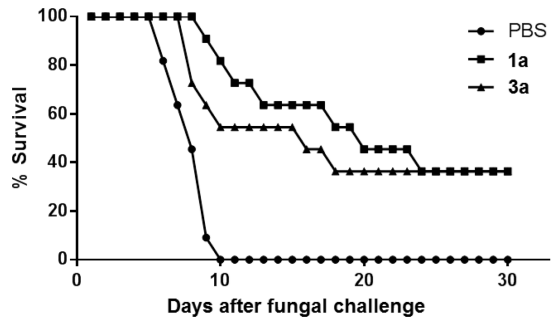

Figure 5. Survival rates of mice immunized with **1a** and **1c** or with PBS (the negative control) after iv injection of a lethal dose of *C. albicans* (7.5×10^5 cells per mouse and 11 mice per group).

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and the animal survival rate was about 82% for **1a** and 55% for **3a** on day 10. At the end of the experiment (30 days after fungal challenge), the survival rate for mice immunized with **1a** and **3a** was 37%, suggesting effective protection of the mice from *C. albicans* challenge. The results unambiguously confirmed that conjugates **1a** and **3a** elicited functional immunity that could effectively protect mice from *C. albicans*-caused infection. Moreover, **1a** provided better protection against *C. albicans* than **3a** at the beginning of infection, which was consistent with the discovery that **1a** elicited higher titers of antibodies than **3a**, but these two vaccines had similar long-term protection against *C. albicans* infection.

Conclusion. To assist in the rational design and optimization of β-glucan-based antifungal vaccine, it is crucial to have detailed information about the structure−antigenicity relationship of β-glucans. For this purpose, synthetic oligo-β-glucans and their conjugates can be especially useful, as they not only contain structurally homogeneous and defined antigens to facilitate structure−activity relationship study but also have other advantages as mentioned above, when compared to vaccines consisting of heterogeneous polysaccharides from natural sources. In this work, three oligo-β-glucans with different branches were coupled with KLH, and the resultant conjugates were evaluated in mice to gain insights into the impacts of side chains in β-glucans on their immunologic properties and to help identify the appropriate antigens for antifungal vaccine design.

The KLH conjugates of all three synthetic branched oligo-β-glucans elicited strong IgG antibody responses, indicating an antibody class switch that is associated with long-lived antibody-mediated protection and T cell-dependent immunity,[18] which is highly desirable for prophylactic vaccines. The results obtained here and in a previous study[29] suggested that **1a**−**3a** elicited a similar pattern and strength of immune responses as the KLH conjugate of an optimized linear oligo-β-glucan, that is,, octa-β-glucan. In the previous paper, we only reported the results with Titermax Gold as the adjuvant, but we also evaluated the linear oligo-β-glucan−KLH conjugates in the presence of CFA, which gave very similar results. Thus, it was concluded that branched oligo-β-glucans should be similarly at least as promising antigens as, if not better than, linear oligo-β-glucans. It was also revealed that antibodies induced by **1a**−**3a** could recognize and bind to natural β-glucans and fungal cells. Most importantly, **1a** and **3a** elicited protective immunities against systemically administered lethal *C. albicans* in mice. The immunologic results of **1a**−**3a** were similar to that of the Lam−CRM_{197} conjugate,[16] even though a more immunogenic carrier protein, CRM_{197}, was used to construct this Lam conjugate. It is worth mentioning that there were differences in experimental details between our and the reported studies. Nonetheless, our results have proved that branched oligo-β-glucans can be useful for the design of functional antifungal vaccines.

Conjugates **1a** and **2a** provoked stronger IgG antibody responses than **3a**, suggesting that both the structure and size of the side chains in branched oligo-β-glucans might have an impact on their immunogenicity. Nonetheless, both **1a** and **3a** were confirmed to elicit protection against *C. albicans* infection, and their long-term protection rates were similar. In contrast, the CRM_{197} conjugate of an oligo-β-glucan with two β-linked glucose units as branches did not elicit obvious protection against fungal infection,[23] even though it did provoke strong immune responses. These results indicated that the number and/or density of side chains in branched oligo-β-glucans might be also important for their immunologic properties. It seemed that to elicit protective immunity, branched oligo-β-glucans needed to carry fewer but longer than monosaccharide branches.

Although **1a** provoked stronger immune responses than **3a**, the two conjugates had similar long-term protection against *C. albicans*. Moreover, the long-term protection rate for **1a** and **3a** (both 37%) was only slightly higher than that (34%) of the KLH conjugate of linear β-octaglucan. The results suggested that so long as the conjugates provoked robust T cell-dependent immunity, they would be able to provide protection against *C. albicans*, even if they had different antibody titers. On the basis of the current results, it is still early to conclude that, as antigens for antifungal vaccine development, branched oligo-β-glucans are significantly better than the optimized linear oligo-β-glucans or that oligo-β-glucans with a β-1,6-linked branch are significantly better than those with a β-1,3-linked branch. Additionally, the readily accessible KLH was used to study the immunologic properties and structure−antigenicity relationships of the synthesized oligo-β-glucans to provide a proof of concept, but KLH is not the best carrier protein for antifungal vaccines. We expect that if more immunogenic carrier proteins, such as CRM_{197} or tetanus toxoid, are utilized to conjugate with the oligo-β-glucans, more potent vaccines and better protection results against fungi may be obtained to provide better understanding about oligo-β-glucans. Subsequently, we plan to couple the synthetic linear and branched oligo-β-glucans with tetanus toxoid or CRM_{197} and study them as vaccines, which will provide more information about their structure−activity relationship and identify the best vaccines for clinical application.

In conclusion, three oligo-β-glucans with different branches were shown to elicit robust, β-glucan-specific, and protective immunities against *C. albicans* in mice. Branched oligo-β-glucans were identified as promising antigens for antifungal vaccine development. We have also revealed that the branches in β-glucans had an impact on its immunologic properties. Thus, it is rational to perform additional systematic studies by using redefined vaccines composed of the proper carrier protein and linear or branched oligo-β-glucans to gain more insights into their immunologic properties and functional mechanisms.

METHODS

Preparation of HSA/KLH−Oligosaccharide Conjugates. Each synthetic oligosaccharide (5.0 mg) was dissolved in a mixture of DMF and 0.1 M PBS (4:1, 0.5 mL), and to the solution was added DSG (15 equiv). After the mixture was stirred at room temperature for 4 h, solvents were removed under vacuum. The resultant activated oligosaccharides were separated from excessive DSG through precipitation with EtOAc (4.5 mL) and washing with EtOAc 10 times. The products were mixed with HSA or KLH (in 30:1 molar ratio) in 0.1 M PBS (0.35 mL) with stirring at room temperature for 3 days. The reaction mixtures were applied to a Biogel A0.5 column to remove excessive oligosaccharides with 0.1 M PBS buffer (I 0.1, pH 7.8) as eluent. Fractions containing the glycoconjugates were combined and dialyzed against distilled water for 2 days. The solution was finally lyophilized to afford the glycoconjugates **1a,b**−**3a,b** as white fluffy solids.

Analysis of Carbohydrate Loadings of Conjugates 1a,b−3a,b with the Sulfuric Acid−Phenol Method.[33] Aliquots of a standard D-glucose solution (1 mg/mL) in water were added in 10 dry 10 mL test tubes in 5 μL increments to

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get standard samples containing 0−50 μg of glucose. In the meantime, an accurately weighed sample of a glycoconjugate **1a,b**−**3a,b** (with the estimated glucose content in the 0−50 μg range) and the corresponding protein were added in two other tubes. To the tubes were added 4% phenol (500 μL) and 96% sulfuric acid (2.5 mL). After stirring for 20 min, the solutions were transferred into cuvettes, and their absorptions at 490 nm wavelength (A490) were measured. A sugar calibration curve was created by plotting the A_{490} of standard samples against the glucose contents and was used to calculate glucose content of each conjugate on the basis of its A_{490} after subtraction of the A_{490} of the corresponding protein sample (the blank):

$$\text{carbohydrate loading (\%)} = \text{sugar wt in tested sample/total wt of sample} \times 100\%$$

Analysis of the Carbohydrate Loadings of Conjugates 1a−3a with MS Method. Conjugates **1a**−**3a**, as well as the free carrier protein HSA, were applied to MALDI-TOF MS analysis to get their average molecular weights (MW). The carbohydrate loadings of conjugates were calculated according the following equation:

$$\text{carbohydrate loading (\%)} = (MW_{conjugate} - MW_{HSA})/MW_{conjugate} \times 100\%$$

Immunization of Mice. Each glycoconjugate **1a**−**3a** (2.07, 2.36, and 2.07 mg, respectively) was dissolved in 10× PBS (0.3 mL) and then diluted with water to form 2× PBS solution. Each was mixed with CFA (1:1, v/v, 1.5 mL) according to the manufacturer's protocol to form an emulsion. Each group of five female C57BL/6J mice (Jackson Laboratory) was initially immunized (day 1) via im injection of an emulsion (0.1 mL) containing about 6 μg of the carbohydrate antigen. Thereafter, each mouse was boosted four times on days 14, 21, 28, and 38 by sc injection of the same emulsion. Mouse blood samples were collected via mouse leg veins on day 0 prior to initial immunization and on days 27, 38, and 48 after boost immunizations. Antisera were prepared from the clotted blood samples.

ELISA.[41] ELISA plates were treated with a solution (100 μL) of HSA conjugate **1a**, **2a**, or **3a** (2 μg/mL) dissolved in coating buffer (0.1 M bicarbonate, pH 9.6) at 4 °C overnight. The plates were incubated at 37 °C for 1 h, washed three times with PBS containing 0.05% Tween-20 (PBST), and incubated with blocking buffer containing 1.0% bovine serum albumin (BSA) in PBS at room temperature for 1 h. After washing with PBST three times, to the plates was added 3-fold diluted (from 1:300 to 1:656100) antiserum in PBS (100 μL/well), followed by incubation at 37 °C for 2 h. The plates were washed with PBST and incubated at room temperature for 1 h with 1:1000 diluted solutions of alkaline phosphatase-linked goat anti-mouse kappa, IgG1, IgG2b, IgG2c, IgG3, or IgM antibody (100 μL/well). The plates were developed with *p*-nitrophenylphosphate (PNPP) (1.67 mg/mL, 100 μL) for 30 min at room temperature and analyzed at 405 nm wavelength. The observed optical density (OD) was plotted against antiserum dilution values in logarithmic scale, and the best-fit line was used to calculate antibody titers that were defined as the dilution value at an OD value of 0.2.

Assay of Lam Inhibition on Antiserum Binding to the Synthetic Oligosaccharides. ELISA plates were coated with HSA conjugates **1b**−**3b** (2 μg/mL) dissolved in 0.1 M coating buffer at 37 °C for 1 h. After being washed with PBST three times, the plates were incubated with BSA blocking buffer. The pooled antisera (1:900 dilution) were mixed with serially diluted PBS solutions of Lam (from 0.01 to 200 μg/mL), and the mixtures were added to the plates that were incubated at 37 °C for 2 h, washed, and incubated with 1:1000 diluted solution of AP-labeled goat anti-mouse kappa antibody (100 μL/well) at room temperature for 1 h. The plates were washed, developed with PNPP (1.67 mg/mL, 100 μL) at room temperature for 30 min, and analyzed at 405 nm wavelength.

$$\text{\% inhibition of binding} = (A_{w/o} - A_w)/A_w \times 100\%$$

$A_{w/o}$ is the absorbance without Lam, and A_w is the absorbance in the presence of Lam.

Immunofluorescence Assay. HKCA cells were smeared on IF microscope slides that were dried, washed with PBST, and treated with 3% BSA blocking buffer at 37 °C for 1 h. The slides were incubated with 1:3 diluted (in PBST) antiserum or normal serum at 37 °C for 2 h, followed by washing and incubation with FITC-labeled goat anti-mouse kappa at room temperature for 1 h. The slides were washed, mounted with the Fluoromount aqueous mounting medium, and studied with the Zeiss ApoTome Imaging System using 100*x*/1.30 oil objective lens.

In Vivo Evaluation of 1a and 3a To Protect Mice against *C. albicans* Infection. Each group of 11 female C57BL/6J mice was immunized with an emulsion of **1a** or **3a** (6 μg of carbohydrate antigen per dose) or with PBS (control) on days 1, 14, 21, and 28. Thereafter, *C. albicans* (strain SC5314) cells (7.5×10^5/mouse), harvested from precultured YEPD medium at 28 °C for 24 h, in 200 μL of PBS were iv injected in the mice on day 38. The mice were monitored daily for 30 days after the systemic challenge with *C. albicans* cell.

Note: The animal protocols used for both immunologic and fungal challenge experiments were approved by the Institutional Animal Use and Care Committee of Second Military Medical University.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsinfecdis.5b00104.

MS spectra of HSA conjugates **1**−**3b** (PDF)

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Notes

The authors declare no competing financial interest.

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