Vaccine xxx (2018) xxx–xxx

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

Protective immune response against *Toxoplasma gondii* elicited by a novel yeast-based vaccine with microneme protein 16

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ARTICLE INFO

Article history:
Received 8 December 2017
Received in revised form 7 May 2018
Accepted 14 May 2018
Available online xxxx

Keywords:
Toxoplasma gondii
Microneme protein 16
Yeast-based vaccine

ABSTRACT

Toxoplasma gondii is an obligate intracellular protozoan that can invade all eukaryotic cells and infect all warm-blood animals, causing the important zoonosis toxoplasmosis. Invasion of host cells is the key step necessary for *T. gondii* to complete its life cycle and microneme proteins play an important role in attachment and invasion of host cells. Microneme protein 16 (TgMIC16) is a new protective protein in *T. gondii* and belongs to transmembrane microneme proteins (TM-MIC). The TM-MICs are released onto the parasite's surface as complexes capable of interacting with host cell receptors. In the present study, we expressed the TgMIC16 protein on the surface of *Saccharomyce cerevisiae* (pCTCON2-TgMIC16/EBY100) and evaluated it as a potential vaccine for BALB/c mice against challenge infection with the RH strain of *T. gondii*. We immunized BALB/c mice both orally and intraperitoneally. After three immunizations, the immune response was evaluated by measuring antibody levels, lymphocyte proliferative responses, percentages of $CD4^+$ and $CD8^+$ T lymphocytes, cytokine production, and the survival times of challenged mice. The results showed that the pCTCON2-TgMIC16/EBY100 vaccine stimulated humoral and cellular immune responses. In addition, mice immunized with the pCTCON2-TgMIC16/EBY100 vaccine showed increased survival times compared with non-immunized controls. In summary, TgMIC16 displayed on the cell surface of *S. cerevisiae* could be used as potential vaccine against toxoplasmosis.

1. Introduction

Toxoplasmosis is a worldwide zoonosis caused by the protozoan parasite *Toxoplasma gondii*, an obligate intracellular parasite [1,2]. *T. gondii* can invade all eukaryotic cells and infect all warm-blood animals, causing risks to human health and the development of animal husbandry [3]. Moreover, toxoplasmosis can be fatal in immunocompromised patients, such as those with AIDS and those undergoing immunosuppressive chemotherapy [4–6]. In addition, evidence has shown a relationship between latent toxoplasmosis and serious psychiatric and neurological diseases [7–9]. The main source of parasite transmission to humans, raw or undercooked meat from intermediate hosts containing *T. gondii* tissue cysts, is considered to be of great public health concern [10].

Currently, there is no commercially licensed *T. gondii* vaccine for use in humans and the use of chemoprophylaxis against toxoplasmosis has several limitations and drawbacks. Firstly, due to the development of drug resistance to anti-*Toxoplasma* chemotherapies and regulations around the use of anti-*Toxoplasma* drugs, chemoprophylaxis strategies have become complex and difficult to apply [11]. Secondly, adverse side effects caused by antiparasitic drugs have necessitated the search for new prevention methods. Recently, many attempts have been made to identify new antigens for the development of toxoplasmosis vaccines [12–14]. These studies indicate a bright future for toxoplasmosis vaccine development.

Yeast is generally regarded as a safe organism (GRAS) and is used as a probiotic for the treatment of various diseases. Yeast has been developed for many years for use in live vaccine delivery systems and has advantages associated with both prokaryotic and eukaryotic organisms. Yeast cell wall components, such as β-glucan, chitin, and mannan, are known to have adjuvant potential [15,16]. The first commercialized recombinant vaccine, the hepatitis B vaccine, was produced in the yeast *Saccharomyces cerevisiae*, resulting in extensive development of yeast-based vaccines [17,18]. Although yeast-based vaccines have been used to protect hosts against bacterial, viral, and parasitic infections, for example, Arnold et al. [19] used *Kluyveromyces lactis* expressing VP2 against infectious bursal disease virus, Shin et al. [20] displayed the ApxIIA

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https://doi.org/10.1016/j.vaccine.2018.05.072
0264-410X/

on the yeast surface to immune BALB/c mice against *Actinobacillus pleuropneumoniae*, and we expressed the EtMIC2 on the surface of *S. cerevisiae* strain HAO as a potential oral vaccine for chicken against *E. tenella* infection [21], a yeast-based vaccine against *T. gondii* has never been reported.

Identification of an appropriate antigen as a vaccine candidate is an important step in the development of vaccines. As key proteins involved in invasion, microneme proteins (MICs) share similar conserved domains with proteins of higher eukaryotes, including epidermal growth factor-like domain (MIC3, MIC6, MIC7, MIC8, MIC9 and MIC12), integrin A-like domain (MIC2), apple module (MIC4 and MIC17), thrombospondin-type repeat (TSR) domain (MIC2, MIC12, MIC14 and MIC15), galectin-like domain (MIC1), chitin binding-like domain (MIC3 and MIC8), and microneme adhesive repeat domain (MIC1 and MIC13) [22,23]. MICs such as TgMIC1, TgMIC2, TgMIC2AP, TgMIC3, TgMIC4, and TgMIC6, are involved in host adhesion and invasion and are promising vaccine candidates [24–27]. TgMIC16 is a conserved TM-MIC with a TM domain located close to the C-terminal end and a rhomboid cleavage site-like motif delimiting a very short C-terminal tail. The amino acid sequence of the protein includes an N-terminal predicted signal peptide and six putative TSR domains [28]. In a previous study, we found that rabbit anti-*T. gondii* serum could recognize *T. gondii* microneme protein (TgMIC16) [29] and Liu [30] constructed the DNA vaccine based on TgMIC16 can effectively induce humoral immune response and cellular immune responses against RH strain and PRU strain infection, indicating that TgMIC16 is a potential vaccine candidate of *T. gondii*. In the present study, we developed a vaccine by expressing TgMIC16 on the surface of *S. cerevisiae* and examined its protective efficacy against challenge infection with *T. gondii* RH strain by measuring immune responses in vaccinated BALB/c mice.

2. Materials and methods

2.1. Experimental mice and parasites

Male and female BALB/c mice aged 5–6 weeks were purchased from the Shandong Laboratory Animal Center, China. Mice were allowed access to food and water ad libitum and experimental procedures were conducted according to institutional guidelines for animal ethics.

The *T. gondii* RH strain tachyzoites were propagated by serial passage in human foreskin fibroblast cells (HFF) and maintained in Dulbecco's modified Eagle's medium (DMEM) (Hyclone, China) with 10% fetal bovine serum (FBS) (Gibco, USA) at 37 °C in 5% CO_2.

2.2. Preparation of vaccine

The construction of recombinant plasmids, yeast surface expression of the TSR domain of TgMIC16 and indirect immunofluorescence assays were performed as described previously [31]. Briefly, the bioinformatics software was used to predict the B cell and T cell epitopes of TgMIC16. According to the predicted results, the antigenic epitope region was included in the TSR domain of TgMIC16. The TSR domain of TgMIC16 expression plasmid pCTCON2-TgMIC16 and the empty plasmid pCTCON2 were transformed into *S. cerevisiae* EBY100 strain and the correct transformants were screened on the SDCAA plate. Then the positive transformants screened out were cultured for displaying TgMIC16 protein on the yeast surface. The displayed TgMIC16 protein was detected by the immunofluorescent labeling assay (IFA) using specific anti-TgMIC16 primary antibody and FITC-conjugated goat anti-rabbit secondary antibody. The yeasts displayed TgMIC16 stained with IFA were analyzed using fluorescence microscope.

2.3. Animal experiments

Freshly harvested yeast cells (4×10^8) were resuspended in 1 ml phosphate buffered saline (PBS) and 100 μl was administered to every mouse. BALB/c mice were randomly assigned to six groups (n = 16 per group). Mice in the vaccinated groups were either injected intraperitoneally with heat-killed (60 °C for 1 h) yeast cells (pCTCON2-TgMIC16/EBY100) or orally administered with live pCTCON2-TgMIC16/EBY100. Control mice (four groups) received either empty vector pCTCON2/EBY100 or PBS by oral gavage or intraperitoneal injection. Mice were immunized using this protocol on weeks 0, 2, and 4. Two weeks after the final injection, mice in all groups were challenged intraperitoneally with 500 virulent *T. gondii* RH strain tachyzoites. Blood was collected from the orbital sinus of the mice after immunization. Sera were separated and stored at −20 °C.

2.4. Measurement of antibody responses

T. gondii-specific antibodies in serum were analyzed by ELISA. Briefly, microtiter plates were coated with 200 ng/well TgMIC16 recombinant protein (expressed in *Escherichia coli* made in our lab) overnight at 4 °C and antibodies were detected with horseradish peroxidase-conjugated goat anti-mouse IgG, IgG1, or IgG2a (Abcam, UK). The average optical density at 450 nm (OD_{450}) was measured using an automated microplate reader (MD, USA) after the reaction was stopped by the addition of 2 M H_2SO_4.

2.5. Lymphocyte proliferation assay

Spleens were aseptically removed from mice (n = 3 per group) 2 weeks after the third immunization. Single-cell suspensions were obtained by filtration through a stainless steel mesh. Red blood cells were removed using erythrocyte lysate buffer (Solarbio, China) and splenocytes were resuspended in RPMI1640 medium (Hyclone) supplemented with 10% FBS at a concentration of 2×10^6 cells/ml. The cells (2×10^5 per well) were then plated in a 96-well plate and cultured in the presence of recombinant TgMIC16 (10 μg/ml), Concanavalin A (ConA; 5 μg/ml; Sigma, USA), or medium alone (negative control) for 72 h at 37 °C in 5% CO_2. Then, 10 μl of Cell Counting Kit-8 (Solarbio) was added to each well and incubated for 3 h. The average optical density at 450 nm (OD_{450}) was measured. All assays were performed in triplicate.

2.6. Evaluation of cytokines

Splenocytes from immunized mice were cultured as described above. Cell-free supernatants were harvested and assayed for interleukin (IL)-2 and IL-4 at 24 h, for IL-10 at 72 h, and for IFN-γ at 96 h. The concentrations of IL-2, IL-4, IL-10, and IFN-γ were determined using an ELISA kit (Dakewe Biotech, China) according to the manufacturer's instructions.

2.7. Flow cytometry analysis

Splenocytes were stained with PerCP-conjugatedanti-CD3e antibody, FITC-conjugated anti-CD4 antibody, and PE-conjugated anti-CD8 antibody (BD, USA). Cells were analyzed with a flow cytometer (Beckman Coulter, USA). About the gating strategy, the percent of $CD4^+/CD8^+$ splenocytes refers to gated lymphocytes.

2.8. Statistical analysis

All samples were assessed in triplicate. All data were analyzed by one-way analysis of variance (ANOVA) using SPSS16.0. Multiple comparisons between the groups were performed using Duncan's

Please cite this article in press as: Wang L-j et al. Protective immune response against *Toxoplasma gondii* elicited by a novel yeast-based vaccine with microneme protein 16. Vaccine (2018), https://doi.org/10.1016/j.vaccine.2018.05.072