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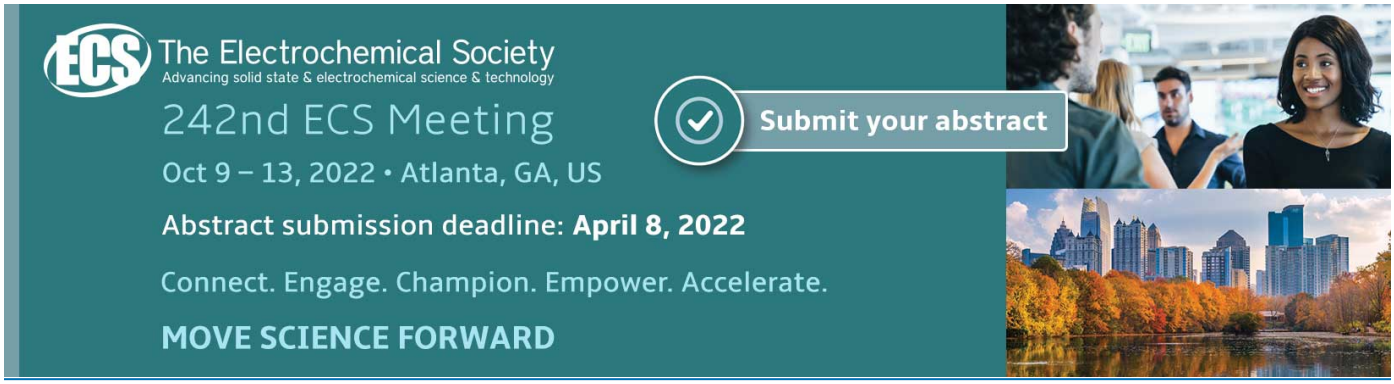

Humoral and cellular immunity in mice immunized with whole recombinant yeast expressing complex NS2B/NS3 protein of dengue serotype 3

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Abstract. A nonpathogenic edible yeast, *Saccharomyces cerevisiae*, has been identified as a vehicle to express many foreign antigens which elicit the immune response in mice. The complex NS2B/NS3 is a protease that represents a prime target for rational drug design for dengue infection. During infection, the NS3 protein is the main target for CD4+ and CD8+ T cell responses, which may be protective. However, no studies have been undertaken evaluating the use of recombinant yeast *Saccharomyces cerevisiae* INVSc1 expressing complex NSB/NS3 protease as a protective antigen against dengue infection. In the present study, we evaluated the humoral and cellular immune response elicited by recombinant yeast compared to wild-type yeast in the mouse model. Intraperitoneal (*i.p.)* administration of recombinant and wild-type yeast at 1 and 25 yeast units into BALB/c mice was used. These studies demonstrated that administration at a low concentration of recombinant yeast at 1 yeast units (YU) significantly elicits antibodies against DENV NS3 antigen. Furthermore, real-time PCR analysis revealed that NS2B/NS3-specific cytocines (TNF-α, IFN-$\copyright$, IL-2) increased with moderate mode compared to wild-type yeast. The results in this study show the potential of recombinant yeast as an edible vaccine platform against dengue infection.

Keyword: yeast, NS2B/NS3 protease, intraperitoneal, humoral, cellular

1. Introduction

Several reasons for using *Saccharomyces cerevisiae* as a vaccine vehicle have been reviewed from several references. For example, it is non-pathogenic, easy to engineer to express multiple antigens in large quantities, heat-killed before administration, and safe in humans, according to several clinical trials [1-4]. Also, recombinant yeast induces strong host immune responses, including cytotoxic T-lymphocyte responses [5].

Saccharomyces cerevisiae is a simple organism to grow and purify, and it is extremely stable. Furthermore, it has been demonstrated that recombinant yeast can elicit a strong immune response in the host in response to nonself-antigens [2, 5]. As a result of these characteristics, S. cerevisiae may be used in cancer immunotherapy regimens in the future. *Saccharomyces cerevisiae* and other yeasts stimulate immune responses by maturing dendritic cells (DCs). The antigen is cross-presented to MHC class I pathways, in addition to the expected MHC class II presentation [6-8]. This yeast has been used as a vaccine carrier in several studies due to its strong immunological response. Vaccination with recombinant S. cerevisiae expressing multiple antigens induces antigen-specific T-cell responses both in vitro and in vivo [6, 9, 10].

Dengue fever is a viral infection spread by mosquitoes (*Aedes aegypti*) that affects an estimated 2.5 billion people worldwide. Dengue viruses cause 50–100 million clinically evident illnesses and up to 50,000 deaths annually [11]. After more than 70 years of research, no effective dengue virus vaccine exists [12].

The NS3 protein (618 aa) contains serine protease and helicase domains required for DENV replication. This protein contains at least 30 T-cell epitopes that are conserved across all four DENV serotypes. Based on these findings, NS3-derived peptides may be targets for reactivated DENV cross-reactive T-cells [13]. It has also been shown that recombinant S. cerevisiae can be used to create a strong T-cell response by expressing NS3 proteins from hepatitis C virus (HCV) and Bluetongue virus (BTV) [3].

In the experiments conducted on the research in this study, a recombinant yeast strain that expressed a DENV NS2B/NS3 protein was put through preclinical studies to test if it could create polyclonal antibody and T cell-mediated immune responses in BALB/c mice.

2. Materials & Methods

2.1. Ethical statement

BALB/c mice were housed and handled in The Center for Pharmaceutical and Medical Technology's (BPPT) animal facility in Indonesia. The experiments were approved by the Medical Faculty of University Indonesia Committee on the Ethics of Animal Experiments (Permit Number: 257/UN2.F1/ETIK/2017) and were conducted according to protocol 17-02-0131.

2.2. Yeast induction and mice immunization

The wild-type and recombinant yeast S. *cerevisiae* INVSc1 containing pYES2CT-NS2B/NS3 expressing complex NS2/NS3 dengue antigen was pre-cultured in minimal media (Yeast Nitrogen Base and Drop-out without Uracil) with 2% glucose to reach a mid-log growth phase at 28 ℃. To overexpressed the NS2B/NS3 antigen, the culture was induced with minimal media with 2% raffinose and 2% galactose. The cells were collected at 72 h after induction by centrifugation at 12000 rpm at 4 °C for 3 min and then washed with phosphate buffer saline (PBS) pH 7.4. The concentration of the yeast cultures was measured at OD_{600} and adjusted to 1 Yeast Unit which corresponds to 1 x 10^7 cells/ml. The yeast cultures were aliquot at 1 YU and 25 YU, stored at 4 ℃ until use. Before immunization, the yeast cells were heat-inactivated at 56 °C for 1 hour.

Five to six-week-old female BALB/c mice were acquired for this study. Groups of 4-5 mice each were intraperitoneally (IP) immunized with 100 μL (containing 1 or 25 YU) of heat-inactivated wild-type yeast, recombinant yeast, and PBS, respectively. These mice received three-times boost immunization with a one-week interval. Sera, bone marrow, PBMC and spleen were collected on day five after the last boosting for humoral and cellular response evaluation.

2.3. Characterization of Humoral Response

Polyclonal antibodies specific to dengue NS2B/NS3 antigen were determined using a standard enzyme-linked immunosorbent assay (ELISA). NS2B/NS3 recombinant antigen at a concentration of 50 ng/mL was coated with 50 mM carbonate buffer, pH 9.4, overnight at 4 ℃. After blocking with PBS Tween 0.05% containing 5% BSA, the coated plate was incubated with all mice sera (diluted 200 times) for 2 hours followed by incubation with secondary antibody goat anti-mouse IgG HRP (1:5000 dilution) (Abcam) for 1 hour at room temperature. The TMB substrate and 2M H_2SO_4 were subsequently added and the intensity of polyclonal antibody was monitored at absorbent 450 nm.

2.4. Characterization of Cellular Response

Total RNA (10 ng for qRT-PCR template) was extracted from PBMC and bone marrow using a one-step phenol/chloroform procedure (TRIZOL®) as directed by the manufacturer (Invitrogen). The tissue was soaked and completely dissolved in TRIZOL solution and 0.25 ml chloroform was added to

1 ml TRIZOL homogenate and thoroughly mixed. The RNA pellet was washed once with 70% ethanol after centrifugation and air-dried for no more than 10 min at room temperature. After dissolving the final RNA pellet in nuclease-free water, the concentration and quality of extracted RNA were determined with A260/280 ratio.

Genes Expression of mouse IFN-©, IL-2, and TNF-α were determined using quantitative real-time PCR. Set primers used in this study were following previous reports [14]. The analysis was performed using a Eco Illumina Real-time PCR System with SensiFast No-ROX One-Step SYBR Green PCR reagents purchased from Bioline. A standard technique called the ΔΔCt method was applied, which is typically used to analyze variations between samples [15]. To account for loading differences, a ΔCt value was calculated for each sample using the Ct value for β-actin. The ΔΔCt value was then calculated by subtracting the corresponding experimental Ct from the ΔCt value for the mice only in the control group (immunized with PBS). The difference between the immunized mice's value and the baseline was calculated as $2^{\Delta\Delta Ct}$.

2.5. Statistical Analysis

ANOVA analysis of variance was used to conduct statistical analyses on the experimental data. A *p*-value < 0.001 was considered as a statistical significance.

3. Results

3.1. Immunization with recombinant yeast induces NS2B/NS3-dengue specific antibody

Several previously published research has demonstrated that recombinant Saccharomyces cerevisiae can induce immune responses in mice [1, 5, 9]. In this study, mice were immunized with 1 YU and 25 YU of wild-type control yeast or recombinant yeast injected *i.p.* on days 0, 7, 14, and 21. Mice were sacrificed 5 days after the last boosting. Sera from all groups were collected and analyzed by ELISA assays against NS3 protein. As shown in Figure 1, immunization with whole recombinant yeast at 1 YU significantly induced specific antibody response (*p<0.001*) compared to wild-type control yeast.

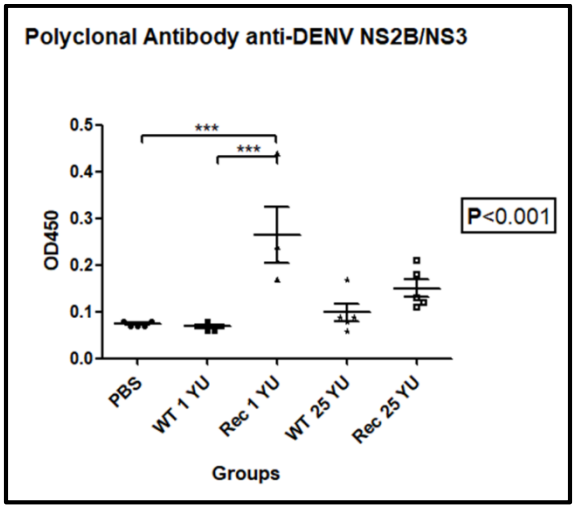

Figure 1. Immunization of BALB/c mice with and without whole recombinant yeast *S. cerevisiae* expressing DENV NS2B/NS3 protein. All groups were immunized with 1 YU or 25 YU at 0, 7, 14, and 21 days. Five days after the last boosting, sera were collected and evaluated using ELISA against DENV NS3 protein. Immunization with whole recombinant yeast at 1 YU significantly induced specific antibody response (p<0.001) compared to wild-type control yeast.

3.2. *The cellular response among all groups*

The mRNA expression level of the cytokines IFN-©, IL-2, and TNF-α, in bone marrow and PBMC of BALB/c mice following immunization with control wild-type yeast and recombinant yeast were evaluated by quantitative real-time polymerase chain reaction (qRT-PCR). The mRNA level of TNF-α was found to increase in whole recombinant yeast groups compared to the wild-type (Figures 2A). In

bone marrow samples, there were no changes in the mRNA level of IFN-© and IL-2 across the groups (Figures 2B, 2C).

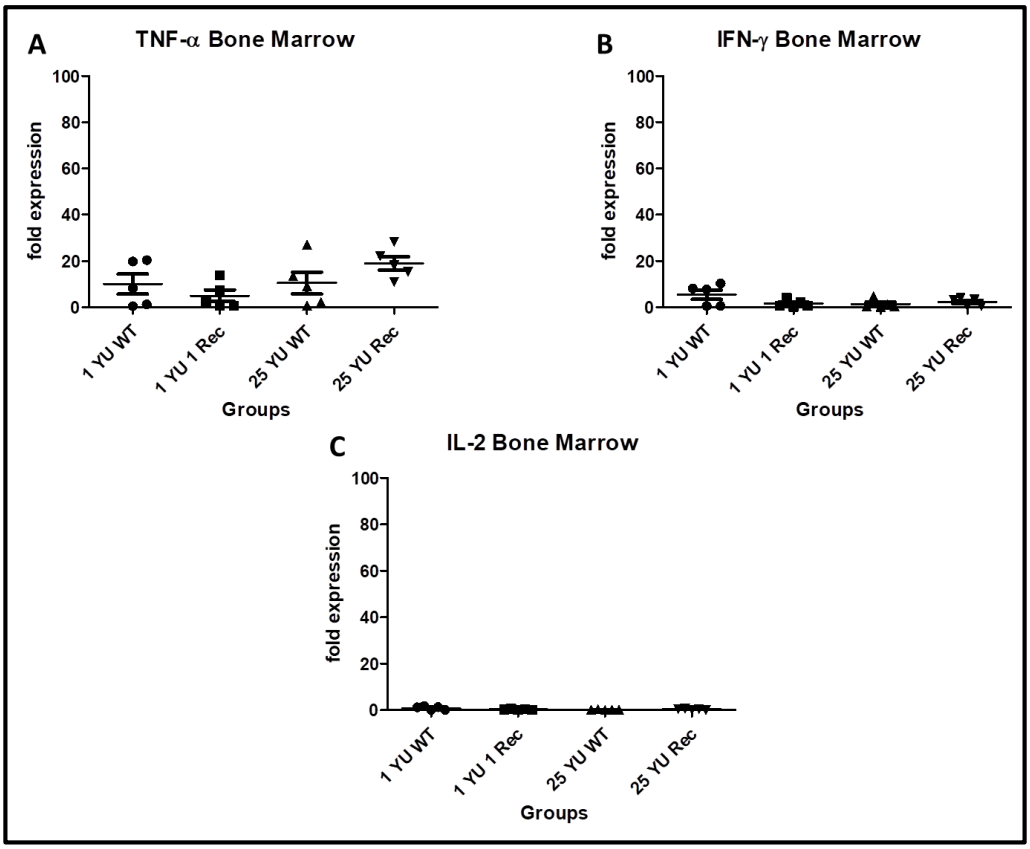

Figure 2. Cytokines gene level analysis was carried out by qRT-PCR from bone marrow. Total RNA was collected from bone marrow and used as a template for cytokine genes. (A) analysis of TNF-*α*, (B) analysis of IFN-©, (C) analysis of IL-2.

In this study, we also examined the mRNA expression level of those cytokines from PBMC. We discovered that mRNA expression levels of IFN-©, IL-2, and TNF-*α* in mice treated with complete recombinant yeast were higher than in animals treated with wild-type yeast (Figure 3).

4. Discussion

Saccharomyces cerevisiae is easily engineered to produce large amounts of one or more antigens, is rapidly grown and purified, and is extremely stable [1]. Since the yeast is heat-killed before administration, the construct is safe. Furthermore, recombinant yeast has been demonstrated to elicit a strong host immune response to nonself-antigens [2, 16]. Nonpathogenic yeast *Saccharomyces cerevisiae* has lately attracted interest as a vaccination carrier for the treatment of infectious diseases [1].

In this study, we sought to determine for the first time whether vaccination with a yeast construct expressing a DENV NS2B/NS3 protein could induce antibody anti-NS3 and antigen-specific T Cell responses. The data presented here show that the BALB/c mice group which was administered with yeast-NS2B/NS3 elicits antibody anti-NS3 especially at low dosage (1 YU). Our findings were consistent with previous studies that found yeast-HBsAg induces significantly higher antibody responses, including IgG, IgG1, and IgG2a [17].

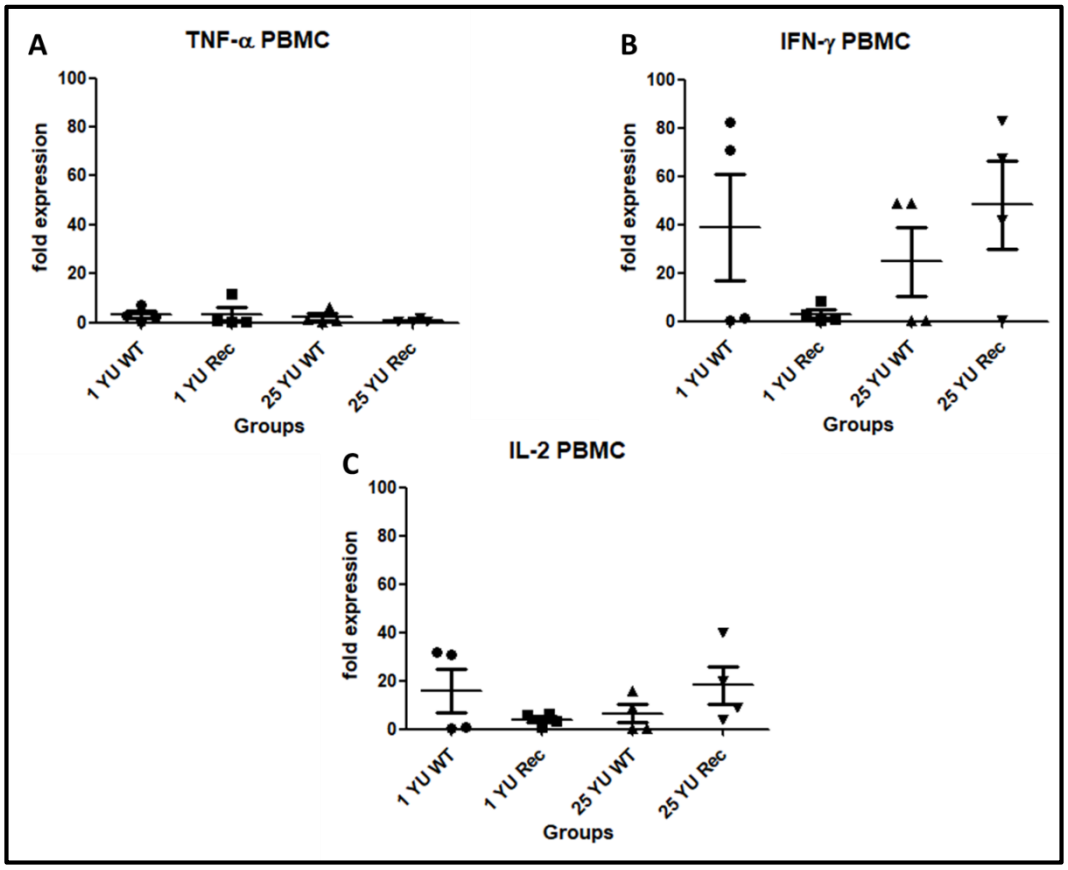

Figure 3. Cytokines gene level analysis was carried out by qRT-PCR from PBMC samples. Total RNA was collected from PBMC and used as a template for cytokine genes. (A) analysis of TNF-*α*, (B) analysis of IFN-©, (C) analysis of IL-2.

Analysis of NS2B/NS3-specific cytokines genes revealed that yeast-NS2B/NS3 is associated with the production of both TNF-*α*, IFN-© and IL-2 in the PBMC, but only TNF-*α* as detected in the bone marrow (Figures 2 and Figures 3). In the previous study, The cellular immunological responses generated by yeast-HCVNS3 injection resulted in immune protection when mice were challenged with tumor cells expressing the HCV NS3 protease constitutively. According to these findings, animals exposed to yeast NS3 sensitize their immune systems and have significantly increased levels of cytotoxic effector cell activity [3]. This observation is similar to that made in CEA-Tg mice, which was previously reported. Numerous cytokine and chemokine mRNAs, as well as genes involved in signal transduction and antigen uptake, were also shown to be rapidly up-regulated using gene expression arrays [18]. TNF-*α*, IFN-© and IL-2 were among the cytokine genes that were upregulated by PBMC in response to whole yeast recombinant. These results indicate the activation of a pro-inflammatory Th-1 type immune response in response to whole yeast recombinant [9].

Briefly stated, we demonstrate that whole recombinant-yeast-based NS2B/NS3 is capable of inducing potent DENV-specific humoral and cellular immunity with a Th1 response profile. However, further analysis should be conducted to evaluate the ability of our construct to neutralize the DENV infection in vivo.

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