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Immune responses induced by heat killed *Saccharomyces cerevisiae:* A vaccine against fungal infection

Min Liu[a,b,c], **Karl V. Clemons**[a,b,c,*], **Marty Bigos**[b], **Izabela Medovarska**[d], **Elmer Brummer**[a,b,c], and **David A. Stevens**[a,b,c]

[a]California Institute for Medical Research, San Jose, CA, United States

[b]Stanford University, Stanford, CA, United States

[c]Santa Clara Valley Medical Center, San Jose, CA, United States

[d]Institute of Chemistry, Glycomed Center, Slovak Academia of Sciences, Bratislava, Slovakia

Abstract

Heat-killed *Saccharomyces cerevisiae* (HKY) used as a vaccine protects mice against systemic aspergillosis and coccidioidomycosis. Little is known about the immune response induced by HKY vaccination, consequently our goal was to do an analysis of HKY-induced immune responses involved in protection. BALB/c mice were vaccinated subcutaneously 3 times with HKY, a protective reagent, and bronchoalveolar lavage fluid, spleen, lymph nodes, and serum collected 2–5 weeks later. Cultured spleen or lymph node cells were stimulated with HKY. Proliferation of HKY-stimulated spleen or lymph node cells was tested by Alamar Blue reduction and flow cytometry. Cytokines from lymphocyte supernatants and antibody to glycans in serum collected from HKY-vaccinated mice were measured by ELISA. The results show that HKY promoted spleen cell and lymph node cell proliferation from HKY-vaccinated mice but not from PBS-vaccinated control mice (all $P < 0.05$). Cytokine measurement showed HKY significantly promoted IFNγ, IL-6 and IL-17A production by spleen cells and lymph node cells (all $P < 0.05$ and $P < 0.01$, respectively). Cytokine production by HKY-stimulated cells from PBS-vaccinated mice was lower than those from HKY-vaccinated ($P < 0.05$). Cytokines in BAL from HKY-vaccinated were higher, 1.7-fold for IFNγ and 2.1-fold for TNFγ, than in BAL from PBS-vaccinated. Flow cytometry of lymphocytes from HKY-vaccinated showed 52% of $CD3^+$ or 56% of $CD8^+$ cells exhibited cell division after stimulation with HKY, compared to non-stimulated controls (26 or 23%, respectively) or HKY-stimulated cells from PBS-vaccinated (31 or 34%). HKY also induced antibody against *Saccharomyces* glucan and mannan with titers 4- or 2-fold, respectively, above that in unvaccinated. Taken together, the results suggested that HKY vaccination induces significant and specific Th1 type cellular immune responses and antibodies to glucan and mannan.

Keywords

Saccharomyces cerevisiae; Vaccines; Fungal infection

[*]Corresponding author at: Division of Infectious Diseases, Santa Clara Valley Medical Center, 751 South Bascom Ave., San Jose, CA, United States. Tel.: +1 408 998 4557; fax: +1 408 998 2723. clemons@cimr.org (K.V. Clemons).

1. Introduction

Numerous efforts have been made in developing antifungal vaccines to meet the increasing medical needs, yet no vaccine is commercially available. Polysaccharide-containing conjugates may be desirable candidates for use in vaccine development [1]. A polysaccharide–protein conjugate that uses the algal glucan, laminarin, was shown to elicit antibodies to β-glucan in fungal cell walls and to induce protection in an infection model of aspergillo-sis and candidiasis [2]. In addition, protection could be passively transferred by immune sera or anti-β-glucan antibodies. Using recombinant *S. cerevisiae* as a vaccine vehicle to express and deliver carcinoembryonic antigens has been shown to have the advantages of less toxicity, good stability, ease of administration along with having inherent adjuvant properties to activate murine den-dritic cells [3,4]. Vaccination resulted in increased gene expression of interleukin (IL)-12, interferon-gamma (IFNγ) and GM-CSF indicating stimulation of cellular immunity [3,4]. We have reported the use of a vaccine preparation using hemolysin expressed in a *Saccharomyces cerevisiae* vector and observed that the empty vector also conferred partial protection in an infection model of coccidioidomycosis [5]. These observations laid the foundation for us to test heat-killed yeast (HKY) as a vaccine candidate against aspergillosis and coccidioidomycosis. We have shown that HKY is effective in protecting CD-1 and BALB/c mice against systemic aspergillosis [6–10], coccidioidomycosis [5], candidiasis [11], and cryptococcosis (T. Majumder, K.V. Clemons, V. Chen, M. Martinez, D. Alvarado, and D.A. Stevens, unpublished data), yet the overall humoral and cellular immune responses after HKY vaccination have not been reported. The aim of this study was to analyze the underlying immune responses associated with HKY-induced protection of the host.

2. Materials and methods

2.1. Animals

Five-week-old male BALB/c mice (Charles River Laboratories, Wilmington, MA) were used in these experiments. Three mice were established per experimental group.

Vaccination—The HKY vaccine was prepared as described previously [5]. HKY (6×10^7 cells) were administered subcutaneously at two dorsal sites (split dose of 0.75 μl each site) once a week for 3 weeks. All animal experiments were done with the approval of the Institutional Animal Care and Use Committee of the California Institute for Medical Research.

2.2. Bronchoalveolar lavage fluid (BALF) collection

Two weeks after the last vaccination, mice were anesthetized with ether and bled from the brachial artery. Lungs were lavaged with 1–2 ml of PBS injected via the trachea as described previously [12]. The lavage fluid (BALF) was centrifuged, and the supernatant stored at −80 °C until analyzed for cytokines. The amount of cytokine present in BALF was expressed as pg/μg total lavage protein.

2.3. Isolation of lymphocytes from tissues

Two or five weeks after the last vaccination, mice were anesthetized and bled from the brachial artery before collection of tissue. Spleens were removed and dispersed through a fine-mesh stainless steel screen in complete tissue culture medium (CTCM; RPMI-1640, 10% fetal bovine serum, penicillin-streptomycin [100 U and 100 μg/ml, respectively]) to obtain single cell suspensions [13]. The cells were pelleted, washed with CTCM, and counted. A total of 10^5 cells/200 μl was added to each well of a 96-well microplate, and incubated at 37°C in 5% CO_2 + 95% air overnight.

The lymph nodes were harvested from brachial, axillary, inguinal, popliteal sites. An average of 8 nodes was dispersed through a fine-mesh stainless steel screen in CTCM [13]. Cells were treated and cultured following the same protocol used for spleen cells.

2.4. Stimulation and cytokine detection in culture supernatant

Spleen cells and lymph node cells from PBS-vaccinated and HKY-vaccinated mice were stimulated with different doses of HKY. Four μg/ml concanavalin A (ConA, Sigma–Aldrich Inc., St. Louis, MO) was used as the mitogen positive control. The optimal dose of ConA and the optimal harvest times to study lymphocyte proliferation and cytokine production were derived from preliminary experiments (not shown). For cytokine assay in spleen and lymph node, cytokine responses were assayed from mouse lymphocytes isolated at two different time points, 2 or 5 weeks after the last dose of HKY immunization. Spleen or lymph node cells were stimulated and cultured for 24 h or 5 d, respectively, at 37 °C in 5% CO_2 + 95% air; the supernatant of the cell culture was used for cytokine analysis. Because the maximum response for each cytokine differed at the two time points in some assays, for ease of presentation, data from the maximum response time has been selected for presentation.

2.5. Enzyme-linked immunosorbent assay (ELISA)

Supernatant fractions of cell culture were centrifuged at 1000 × *g* for 5 min, divided into microtubes and stored at −80 °C until use. To simultaneously test the cytokine expression profile, we performed a fast multiplex detection of cytokines in the cultured supernatant using a multi-protein profiling ELISA kit (Superarray Biosciences, Frederick, MD). The expression level of each cytokine was measured using specific ELISA kits for INFγ, IL-6 (BD Biosciences, Franklin Lakes, NJ) and IL-17A (Superarray Biosciences, Frederick, MD). The microplates were read at 450 nm and 550 nm, the optical imperfections in the microplate were corrected by subtracting 550 nm values from 450 nm values of absorbance.

2.6. Cell proliferation assay

Lymphocyte proliferation was tested after 3 d (spleen cells) or 7 d (lymph node cells) of incubation with the ConA or HKY stimulus. Twenty μl Alamar Blue was used as an indicator in each well. After 8 h of incubation, the microplate was read at 570 and 600 nm in plate reader. Proliferation was defined by:

$$\text{stimulation index} = \frac{A_{570} - (A_{600} \times R_0) \text{ for test well}}{A_{570} - (A_{600} \times R_0) \text{ for non- stimulated control well in each antigen- stimulated group}}$$

where $R_0 = A_{570}$ (absorbance of oxidized form at 570 nm)/A_{600} (absorbance of oxidized form at 600 nm).

2.7. Determination of mononuclear cell proliferation by flow cytometry

Spleen cells from PBS-vaccinated and HKY-vaccinated mice were isolated 4 weeks after the last vaccine dose. The mononu-clear cells were isolated using Histopaque density centrifugation (Sigma–Aldrich, Inc., St. Louis, MO). Cells were washed by centrifugation and suspended in PBS with 0.1% BSA (w/v) to 10^6 cells/ml. Carboxyfluorescein diacetate succinimidyl ester (CFSE, Molecular Probes, Eugene, OR) was added to a final concentration of 0.5 μM and incubated at 37 °C for 10 min. Cells were washed by centrifugation three times with ice-cold CTCM, counted and cultured at 10^6 cells/well at 37 °C in 5% CO_2 + 95% air. Cell cultures were stimulated with 4×10^3, and 4×10^4 and 4×10^5 HKY cells for 64 h. Cells were collected and washed three times by centrifugation at 200 × *g* with PBS. Cells were stained with Live/Dead Fixable Aqua Dead Cell Stain (Invitrogen) for 30 min for gating. Cells were collected by centrifugation and washed three times with PBS/0.1% BSA. To stain cell surface antigens, cells were incubated for 30 min with 1 μM Pacific Blue anti-mouse CD3 (eBioscience), PE-Cy5 (phycoerythrin-cyanine dye 5) labeled anti-mouse CD4 (eBioscience), and PE-Texas Red labeled rat anti-mouse CD8α (Invitrogen). After washing three times at 250 × *g* with PBS–0.1% BSA, cells were analyzed by flow cytometry (LSRII flow cytometer, BD Bioscience).

2.8. Antibody titration

Serum was isolated from blood collected 2 weeks after the last vaccination from PBS or HKY-vaccinated mice. Antibody titers in the serum for glucan and mannan were determined by the optimal ELISA method described previously [14], and expressed as the reciprocal of the highest positive dilution. Final titers are expressed as the mean ± SD of triplicate determinations.

2.9. Statistical analysis

Proliferation and cytokine expression were measured from three experiments; data are presented as a mean plus or minus standard error of triplicate samples. Statistical analysis was done using Student's *t*-test.

3. Results

3.1. HKY stimulates proliferation of spleen and lymph node cells

We sought to evaluate whether HKY induced lymphocyte proliferation in a specific or nonspecific manner. Mice were vaccinated with HKY or PBS and cells harvested 5 weeks after the last dose. Cell proliferation, as measured by Alamar Blue reduction, was assessed for spleen and lymph node cells in response to HKY stimulus. Only the mitogen, ConA,

promoted significant proliferation of spleen cells from PBS-vaccinated mice ($P = 0.02$) compared to its non-stimulated control (Fig. 1A); HKY did not induce significant proliferation. For spleen cells from HKY-vaccinated mice, all doses of HKY promoted significant proliferation compared to the non-stimulated control ($P = 0.002$, $P = 0.01$, $P = 0.02$, respectively); stimulation with 4×10^4 cells of HKY trended toward significant induction of proliferation. Proliferation of the cells from HKY-vaccinated mice was significantly greater than that induced in cells from the PBS-vaccinated mice stimulated with 4×10^5 HKY ($P = 0.049$).

Lymph node cells from PBS-vaccinated mice responded similarly to spleen cells in that only ConA promoted significant proliferation ($P = 0.01$) (Fig. 1B). Interestingly, these lymph node cells exposed to HKY stimulus showed a non-significant reduction in proliferative response. In contrast, lymph node cells from HKY-vaccinated mice showed a dose-responsive proliferative response, compared to the non-stimulated control, with 4×10^4 and 4×10^5 cells of HKY promoting significant proliferation ($P = 0.01$, $P < 0.01$) (Fig. 1B). Proliferation of the lymph node cells from HKY-vaccinated mice stimulated with doses of 4×10^4 or 4×10^5 HKY was significantly higher than that induced by the same doses of HKY for cells from the PBS-vaccinated mice ($P = 0.049$).

3.2. HKY stimulates IFNγ, IL-6 and IL-17A production in spleen cells

To obtain a global picture of cytokine expression profiles, a multi-cytokine array with the supernatant from cultured spleen cells or lymph node cells was performed. Array results showed that expression of IFNγ, IL-6 and IL-17A was up-regulated after stimulation of the cells with HKY (data not shown). Therefore, each of these cytokines was further quantified using single cytokine ELISA.

IFNγ—IFNγ production by spleen cells was tested 5 weeks after the last vaccination (Fig. 2A). In spleen cells from PBS-vaccinated mice, compared to the unstimulated control, all doses of HKY and ConA (4 μg/ml) significantly promoted IFNγ expression. The highest level of IFNγ was found in HKY (4×10^4 HKY per well) stimulated cell culture supernatant (640 pg/ml). In spleen cells from HKY-vaccinated mice, compared to the unstimulated control, all doses of HKY significantly stimulated IFNγ production (all $P < 0.05$) and the highest level was produced by cells stimulated with 4×10^5 HKY (11,403 pg/ml). IFNγ induction by all doses of HKY used to stimulate the cells from HKY-vaccinated mice was significantly higher than was induced in cells from PBS-vaccinated mice (all $P < 0.01$).

IL-6—IL-6 production by spleen cells was assayed 5 weeks after the last dose of HKY (Fig. 2B). Compared to the non-stimulated control cells, HKY stimulation (4×10^4 or 4×10^5 HKY per well) (both $P < 0.01$) of spleen cells from PBS-vaccinated mice and ConA (4 μg/ml) significantly promoted IL-6 expression ($P = 0.048$). The highest level of IL-6 was produced by HKY (4×10^4 HKY) stimulated cells (171 pg/ml). After 24 h of stimulation of spleen cells from HKY-vaccinated mice, compared to the unstimulated control, all doses of HKY and ConA (4 μg/ml) significantly stimulated IL-6 production. The highest level of IL-6 was produced by cells stimulated with HKY (4×10^5 HKY per well) (1177 pg/ml). IL-6 induction by different doses of HKY (4×10^3, 4×10^4, or 4×10^5 HKY) by cells from

HKY-vaccinated mice was significant compared to stimulation of cells from PBS-vaccinated mice (all $P < 0.01$).

IL-17A—IL-17A production by spleen cells was tested 5 weeks after the last dose of HKY vaccination (Fig. 2C). For spleen cells from PBS-vaccinated control mice, compared to the unstimulated control, the 4×10^4 dose of HKY or ConA (4 μg/ml) significantly promoted IL-17A production (all $P < 0.05$) and for 4×10^4 HKY stimulated cell culture supernatant was 182 pg/ml. In spleen cells from HKY-vaccinated mice, 4×10^4 or 4×10^5 doses of HKY or 4 μg/ml ConA significantly stimulated IL-17A production, while 4×10^6 dose of HKY did not significantly induce IL-17 A production ($P > 0.05$); 4×10^5 HKY stimulated cells to produce the most IL-17A (576 pg/ml). IL-17A induction by different doses of HKY (4×10^4 or 4×10^5) in the cells from HKY-vaccinated mice was significant compared to those induced by equivalent doses of HKY by cells from PBS-vaccinated mice (all $P < 0.01$).

3.3. HKY stimulates cytokine production in lymph node cells

IFNγ—IFNγ expression by stimulated lymph nodes cells was assayed 5 weeks after the last HKY vaccination (Fig. 3A). For lymph node cells from PBS-vaccinated mice, compared to the unstim-ulated control, 4×10^5 of HKY and ConA (4 μg/ml) significantly promoted IFNγ expression (both $P < 0.01$). The highest level of IFNγ was produced by cells stimulated with 4×10^5 HKY per well (296 pg/ml). For lymph node cells from HKY-vaccinated mice, 4×10^4 and 4×10^5 HKY per well significantly stimulated IFNγ production ($P = 0.002$, $P = 0.005$, respectively). The highest level of IFNγ was produced by cells stimulated with 4×10^4 HKY (540 pg/ml). IFNγ induction in the cells from HKY-vaccinated mice by different doses of HKY (4×10^3, 4×10^4, and 4×10^5 HKY per well) were significantly higher than those induced by the equivalent doses of HKY for cells from PBS-vaccinated mice (all $P < 0.01$).

IL-6—IL-6 expression in lymph node cells was assayed 2 weeks after the last HKY vaccination (Fig. 3B). For lymph node cells from PBS-vaccinated mice, only ConA (4 μg/ml) significantly promoted IL-6 production. However, for lymph node cells from HKY-vaccinated mice, compared to the unstimulated control, all doses of HKY or ConA (4 μg/ml) significantly stimulated IL-6 production (all $P < 0.05$). The highest level of IL-6 was produced by cells stimulated with 4×10^5 HKY (660 pg/ml). IL-6 production, after different doses of HKY (4×10^3, 4×10^4, and 4×10^5 HKY per well), by cells from HKY vaccinated mice was significantly higher than those from cells from PBS-vaccinated mice stimulated with equivalent doses of HKY (all $P < 0.01$).

IL-17A—IL-17A production by lymph node cell was assayed 2 weeks after the last dose of HKY (Fig. 3C). No HKY dose significantly promoted IL-17A production by lymph node cells from PBS-vaccinated mice, compared to the unstimulated control, whereas ConA (4 μg/ml) significantly induced IL-17A ($P < 0.01$). In contrast, all doses of HKY or ConA significantly stimulated IL-17A production by lymph nodes cells from HKY-vaccinated mice (all $P < 0.01$). Cells stimulated with 4×10^5 HKY per well produced 8848 pg/ml of IL-17A. IL-17A from cells from HKY vaccinated mice induced by different doses of HKY

(4×10^3, 4×10^4, and 4×10^5 HKY) was significantly higher than those induced by the equivalent doses of HKY for cells from PBS-vaccinated mice (all $P < 0.01$).

3.4. HKY stimulates IFNγ and TNFα in bronchoalveolar lavage fluid (BALF)

Expression of cytokines in BALF was tested in comparison to the PBS-vaccinated control group (Fig. 4). Significant levels of IFNγ and TNF-α were detected in BALF from HKY-vaccinated mice in comparison with PBS-vaccinated mice ($P < 0.01$). BALF from HKY-vaccinated mice contained 1.594 pg IFNγ/μg total lavage proteins, a 1.73-fold increase compared to BALF from PBS-vaccinated. Similarly, BALF from HKY-vaccinated mice contained 0.992 pg TNFγ/μg total lavage proteins, which was 2.11-fold higher than in BALF from PBS vaccinated mice ($P = 0.007$).

3.5. Flow cytometry analysis of cell proliferation

To determine the subpopulation of spleen lymphocytes proliferating in response to HKY, we used a CFSE-based method to stain individual cells that had undergone cell divisions coupled with different cell surface markers. An example of the gating sequence used for Flow cytometry analysis of cell proliferation by cultured lymphocytes is shown in Fig. 5. HKY-vaccinated mice showed that approximately 52% of $CD3^+$ cells exhibited cell division after stimulation with 4×10^5 HKY. In comparison, 26% of the non-stimulated control cells or 31% of HKY-stimulated cells from PBS-vaccinated mice proliferated (Fig. 6). Similarly, 56% of $CD8^+$ cells exhibited cell division after stimulation with 4×10^5 HKY, in comparison to 23% for non-stimulated control cells or 33.5% of HKY-stimulated cells from PBS-vaccinated mice (Fig. 7). We did not observe cell division of $CD4^+$ cells after HKY stimulation.

3.6. Antibody response induced by HKY

To assess whether HKY induced protection might result from an antibody response, we measured specific anti-mouse IgG titers against glucan and mannan by ELISA assay using serum collected from HKY-vaccinated mice. Antibody assay against glucan and mannan from *Saccharomyces* showed titers 1600 ± 38 and 400 ± 36, respectively, which was 4 or 2 times above that in PBS-vaccinated. Statistical analysis indicated a significant difference ($P < 0.01$) between the mean antibody titers of HKY-vaccinated and PBS-vaccinated mice for both tested antigens.

4. Discussion

Our goal in this study was that of examining the immune response induced by vaccination with HKY that might in part explain its utility as a vaccine against experimental coccidioidomycosis and aspergillosis. To determine ability of HKY in triggering cell-mediated immune responses, we performed cellular proliferation assays that showed HKY were effective in stimulating cell proliferation in cells from HKY-vaccinated mice, but not from sham-vaccinated mice. These results indicated that HKY vaccination induced specific lymphocyte responses to HKY antigens and an expansion of a population of primed lymphocytes indicative of an adaptive immune response. Presumably these responses are

directed against protein and/or glucan antigens shared among *Saccharomyces*, *Aspergillus*, and *Coccidioides.*

Our results from multiple-color flow cytometry staining of lymphocyte populations indicated that HKY-stimulation of cells from HKY-vaccinated mice resulted in proliferation of $CD3^+$ and $CD8^+$ T cells, but not $CD4^+$ cells. There are reports showing that proinflammatory cytokines, such as IFN-γ, are also produced by different immune cell populations, including $CD8^+$ and $CD3^+$ cells in response to various fungal infections or antigens [15–18]. In addition, there is evidence from other fungi showing that $CD4^+$ T cells are dispensable for vaccine-induced resistance against experimental pulmonary infections [16,19,20]; instead, antigens are capable of activating CD8 memory cells to produce TNFα and IFNγ for protection. These results further support our conclusion that the immune response to HKY-vaccination results in adaptive rather than innate immune response. $CD8^+$ T cells recognize pathogen-derived antigens complexed with major histocompatibility complex (MHC) class I molecules on the surface of antigen presenting cells (APCs). Usually dendritic cells or macrophages are APCs and secrete cytokines such as TNF and IFNγ to contribute toward antimicrobial defense [21]. Priming of antigen-specific $CD8^+$ T cells results in proliferation and contraction into stable memory populations [21]. HKY vaccination-induced protective efficacy may depend on $CD8^+$ and $CD3^+$ cell function to produce defensive cytokines.

IFNγ, IL-6 and IL-17A were elevated in the supernatant of lymphocyte cell cultures from HKY-vaccinated mice in response to HKY. The low level of cytokine production occasionally observed in response to HKY in PBS-vaccinated mice could represent a low level of cross-reactivity to environmental antigens. Thus, HKY-vaccination primed lymphocytes for Th-1 type of cytokine production. Heat-killing of HKY causes denaturation of proteins, which tends to shift the host response to a Th1 type [22].

IFNγ is a cytokine necessary for activation of phagocytes to kill several different fungal pathogens, potentiating the oxidative responses to cause hyphal damage, and enhances monocyte function against a variety of fungi [23] including *Aspergillus* [24] and *Coccidioides* [25]. Similarly, IL-6 is a pro-inflammatory cytokine and a potent activator of T-lymphocytes and neutrophils [26]. IL-6 was shown to potentiate host defense against coccidioidomycosis [27], and IL-6-deficient mice are more susceptible to invasive pulmonary aspergillosis [28]. IL-6 in conjunction with TGF-γ induces the differentiation of naïve T lymphocytes into IL-17-producing Th17 cells [29]. IL-17 recently has been identified as a T cell-derived cytokine that is involved in host defense response against bacteria and fungal infection [30,31]. IL-17 has been shown particularly important in resistance to *Aspergillus* infection in an animal model [28] and is also considered critical to a protective response against candidiasis [32,33]. In human cells, IL-17 expression was induced above baseline by stimulation with whole *S. cerevisiae* cells proposed to be a result of the interaction between mannan and mannan receptor (MR) triggering IL-17 production [34]. In addition, HKY-stimulation of lymphocytes might also result in elevated levels of IL-17 from exposed β-glucan stimulation of macrophages or mononuclear cells (i.e., innate immune cells), which further prime Th17 cells [35].

Macrophages are the first defensive line against fungal infection, particularly pulmonary infection, and the antimicrobial function of bronchoalveolar macrophages is regulated by proinflammatory cytokines. TNFα and IFNγ were measurable in the BALF collected from non-challenged lungs of mice 2 weeks after the last HKY vaccination and significantly higher in concentration in BALF from HKY-vaccinated mice compared to BALF from PBS or unvaccinated mice. Both IFNγ and TNFα play key roles in pulmonary macrophage activation and destroying different infectious bacterial [36] and fungal agents, including *Blastomyces*, *Paracoccidioides*, *Histoplasma*, and *Aspergillus* [23,36,37]. The expression of IFNγ and TNFα in BALF suggests that HKY-vaccination induces a specific cytokine response that would be essential for the host defense against fungal infection. Future studies using models of pulmonary aspergillosis or coccid-ioidomycosis infection will be essential in determining whether immune responses to HKY vaccination expressed through the pulmonary immune system, such as the IFNγ and TNFα in the BALF that we demonstrated exemplifies, will impart resistance to infection. In line with the results of our study, the capability of *S. cerevisiae* to induce proinflammatory cytokine mRNA expression and cytokine secretion also has been shown in other studies [34,38].

It remains to be determined which particular antigens evoke protective recall responses in HKY-vaccinated mice. Fungal cell wall components, glucan and mannan, are predominant antigens common to many fungi. These components have immunostimulatory properties, often associated with the innate immune response, as a result of recognition of the pathogen-associated molecular patterns by cellular receptors such as mannose receptor and dectin-1, which in turn triggers phagocytosis, respiratory activity and induction of cytokine gene expression [39]. However, glycans can also act as antigens and are usually classified as T-independent antigens evoking only a humoral response [40].

Glucans and mannans can induce antibodies that confer protection to mice against aspergillosis and candidiasis [2,41–48]. Our antibody assay against *Saccharomyces* glucan and mannan showed that HKY-vaccination induced significant IgG titers, which were 4 or 2 times higher, respectively, than those in PBS-vaccinated mice. B-cells, unlike T-cells, are stimulated directly by antigen and do not depend on antigen presentation by APCs. B-cells bind antigen by surface IgM resulting in signaling pathways leading to antibody producing plasma cells.

We have shown here that HKY-vaccination induces a specific adaptive and comprehensive immune response in mice. These results are supportive of those of our *in vivo* studies, where the protective efficacy of HKY vaccination against coccidioidomycosis, candidiasis and aspergillosis has been repeatedly observed [5–11]. However, the components of HKY responsible for the cross-protective response against these infections are not known. Several homologous proteins have been described among *Saccharomyces*, *Aspergillus* and *Coccidioides*. These include the chitinases, *cts1*, in *Saccharomyces*, *cts2* in *Coccidioides*, and chitinases from *Aspergillus* [49]; and the alkyl hydroperoxide peroxidase, *Ahp1*, in *Saccharomyces*, with peroxisomal matrix proteins, *Pmp1*, in *Coccidioides*, and *Aspf3* in *Aspergillus* [50,51]. Further study on homologous proteins and their association with cell wall glycans will contribute to understanding the mechanism of HKY-induced adaptive protection.

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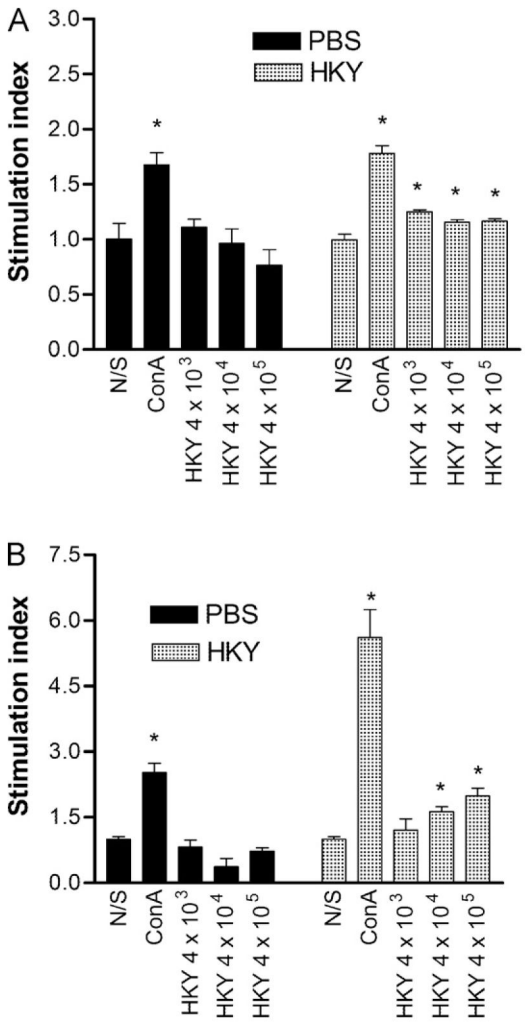

Fig. 1.

Proliferation of spleen and lymph node cells from PBS-vaccinated and HKY-vaccinated mice stimulated with HKY or ConA (4 μg/ml). (A) Spleen cells were stimulated for 3 d with ConA or HKY doses as indicated. (B) Lymph node cells were stimulated for 7 d with ConA or HKY doses as indicated. Alamar Blue was added to each well for additional 8 h to detect proliferation on the basis of reduction of the Alamar Blue. Each bar represents the mean ± SE. An * indicates $P < 0.05$ in comparison with the non-stimulated (N/S) control cells. *Abbreviations*: N/S, non-stimulated; ConA, concanavalin A; HKY, heat-killed yeasts.

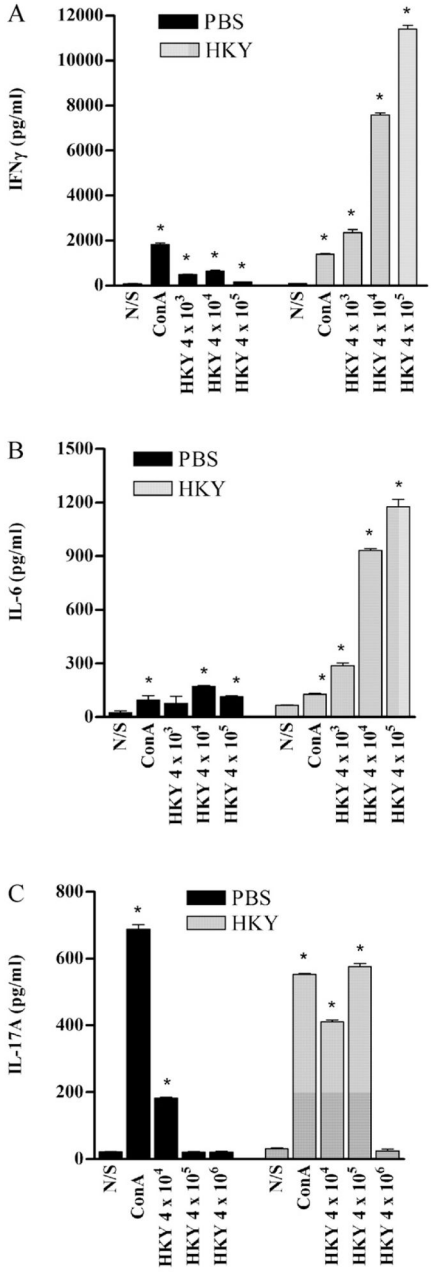

Fig. 2.

Cytokines detected in supernatants of spleen cell culture from PBS- and HKY-vaccinated mice, 5 weeks after last dose of vaccination. Cells were stimulated with different doses of HKY or ConA (4 μg/ml) for 3 d and culture supernatant was collected for ELISA. (A) IFNγ production. (B) IL-6 production. (C) IL-17A production. Each bar represents the mean ± SE. An * indicates $P < 0.05$ in comparison with the non-stimulated (N/S) control cells.

Abbreviations: N/S, non-stimulated; ConA, concanavalin A; HKY, heat-killed yeasts.

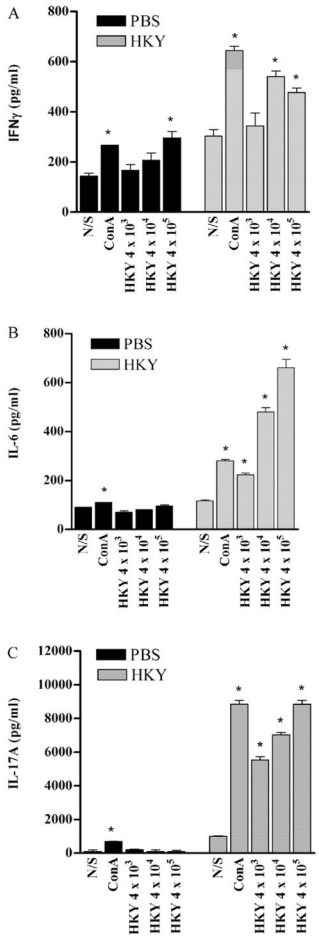

Fig. 3.

Cytokines detected in supernatants of lymph node cell culture from PBS-vaccinated mice and HKY-vaccinated mice, 2 weeks (IL-6, IL-17A) or 5 weeks (IFNγ) after the last vaccination. Cells were stimulated with different doses of HKY or ConA (4 μg/ml) for 3 d. Culture supernatant was collected for ELISA. (A) IFNγ production. (B) IL-6 production. (C) IL-17A production. Each bar represents the mean ± SE. An * indicates $P < 0.05$ in comparison with the non-stimulated (N/S) control cells. *Abbreviations*: N/S, non-stimulated; ConA, concanavalin A; HKY, heat-killed yeasts.

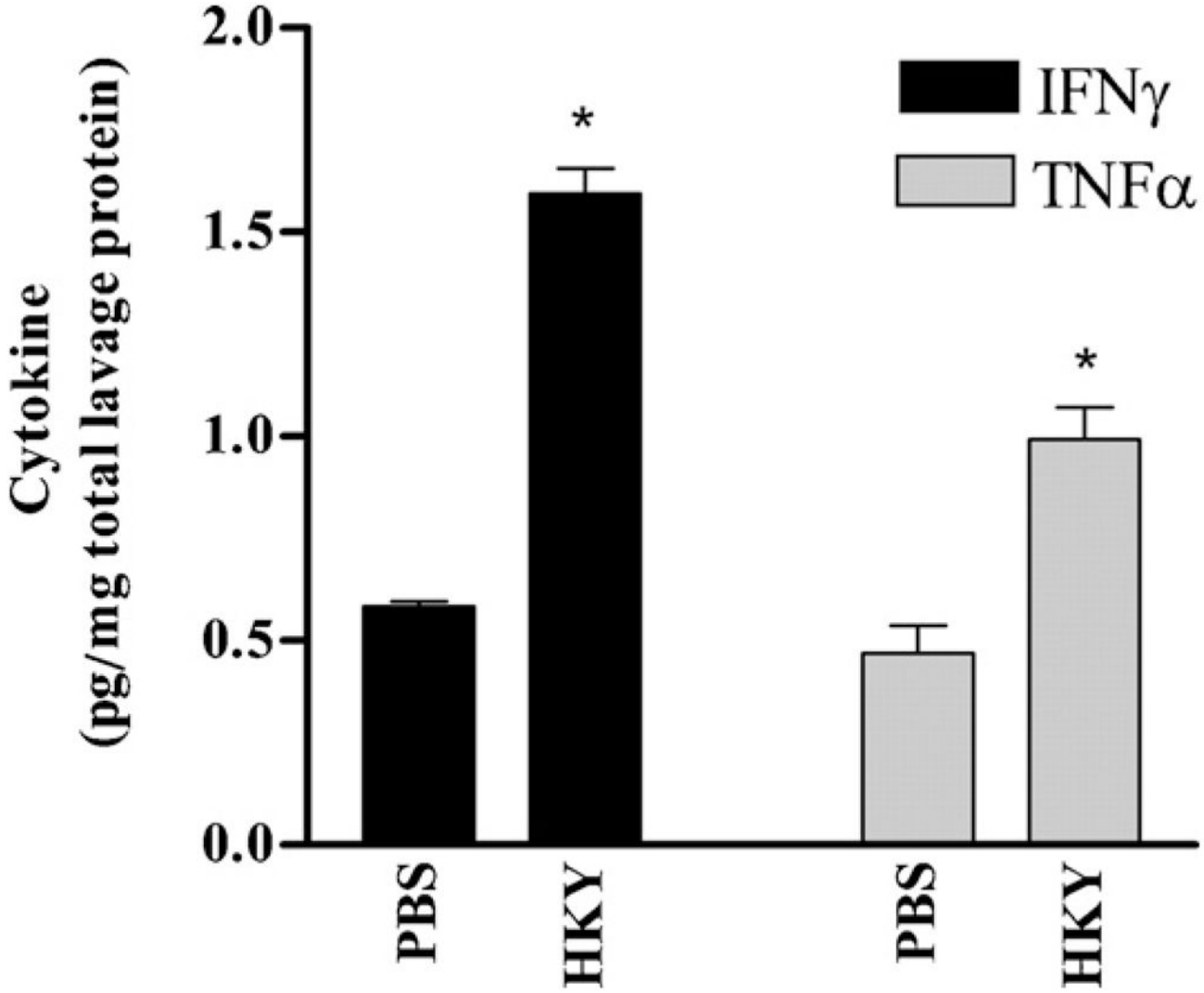

Fig. 4.

IFNγ and TNF-α detected in bronchoalveolar lavage fluid (BALF) from BALB/c mice 2 weeks after the last PBS or HKY vaccination. An * indicates $P < 0.05$ in comparison with PBS-vaccination.

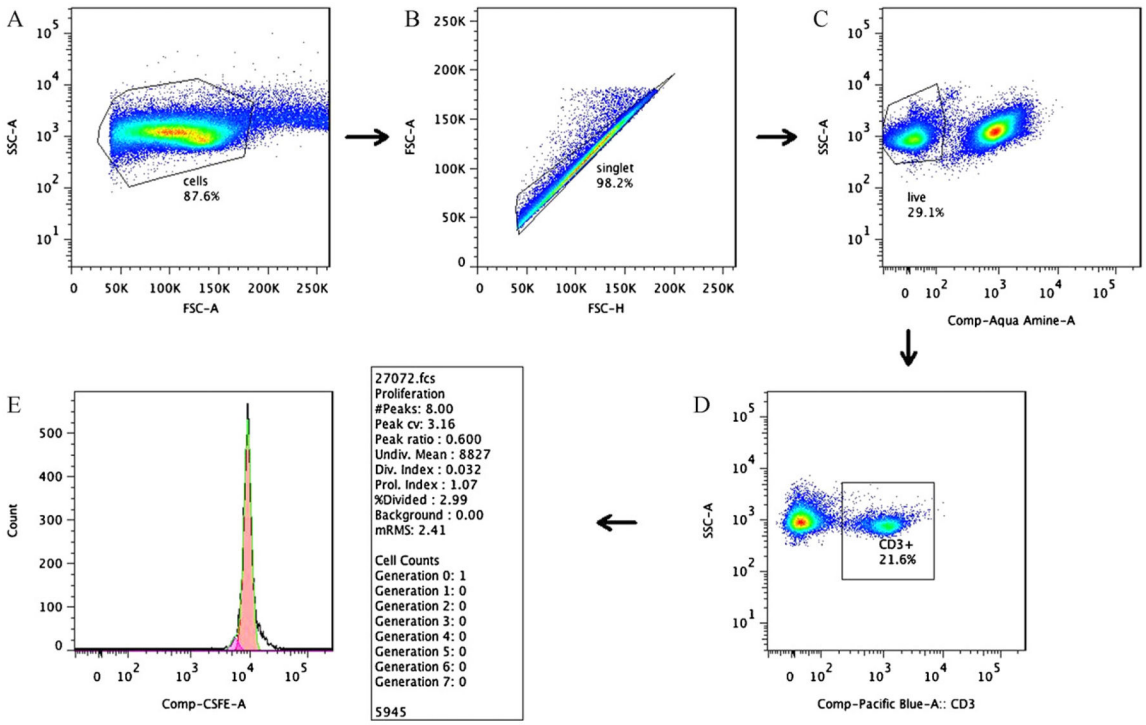

Fig. 5.

Flow chart illustrated the gating sequence used for cell proliferation analysis by multi-color flow cytometry. (A) Gated total population of all stained mononuclear cells, (B) singlet of gated mononuclear cells, (C) gating of aqua-stained mononuclear cells for live cells (i.e., nonstained), (D) gating of live cells in panel C for $CD3^+$ mononuclear cells, and (E) example of a proliferation histogram of $CD3^+$ control cells showing no cell division (dashed line indicates the model generated by FlowJo 7.2.4 software).

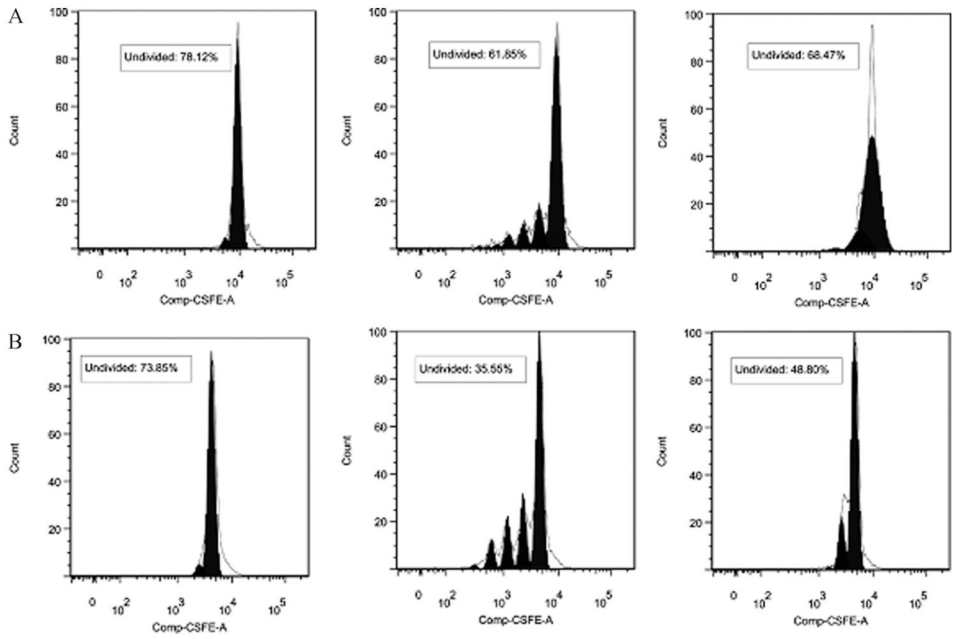

Fig. 6.

Histograms showing proliferation of $CD3^+$ T cells in spleen cells isolated from non-vaccinated (A) and HKY vaccinated (B) mice after *in vivo* stimulation: non-stimulated control cells (left), ConA (middle) and 4×10^5 cells of HKY (right) for 64 h. CFSE profile from gate illustrates fitted model sum (proliferation model according to FlowJo 7.2.4 software, shown by the clear peaks under the black lines) to determine gates for division number (shown by the filled solid peaks in black). The right black peak in each graph represents generation 0 (undivided) lymphocytes. The number of peaks located on the left side of each generation 0 lymphocytes indicates the division numbers undergone by the lymphocytes.

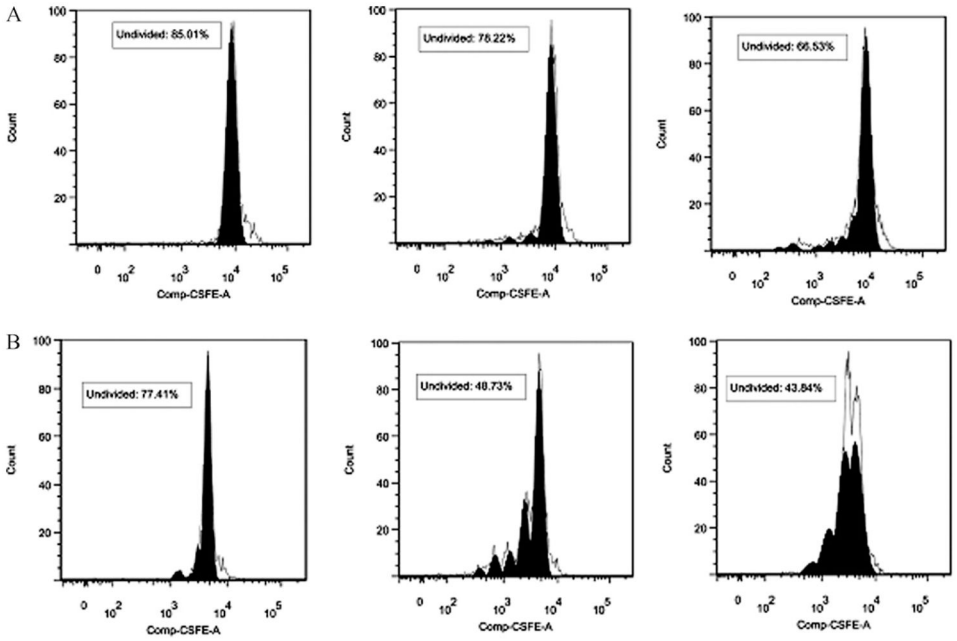

Fig. 7.

Histograms showing proliferation of $CD8^+$ spleen cells isolated from non-vaccinated (A) and HKY-vaccinated (B) mice after *in vivo* stimulation with: non-stimulated cells (left), ConA (middle) and 4×10^5 cells of HKY (right) for 64 h. CFSE profile from gate illustrates fitted model sum (proliferation model according to FlowJo 7.2.4 software shown by the clear peaks under the black lines) to determine gates for division number (shown by the filled solid peaks in black). The right black peak in each graph represents generation 0 (undivided) lymphocytes. The number of peaks located on the left side of each generation 0 lymphocytes indicates the division numbers undergone by the lymphocytes.