

Gut Colonization by *Candida albicans* Inhibits the Induction of Humoral Immune Tolerance to Dietary Antigen in BALB/c Mice

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We previously observed that gut colonization by *Candida albicans* promoted serum antibody response to orally administered ovalbumin in mice. We therefore postulated that *C. albicans* affects oral tolerance induction. The present study tested this idea. BALB/c mice were intragastrically administered with either *C. albicans* (1×10^7) or vehicle, and the colonization was confirmed by weekly fecal cultures. Mice were further divided into two subgroups and intragastrically administered with either ovalbumin (20 mg) or vehicle for five consecutive days. Thereafter, all mice were intraperitoneally immunized with ovalbumin in alum. In mice without *C. albicans* inoculation, ovalbumin feeding prior to immunization significantly suppressed the increase in ovalbumin-specific IgE, IgG1 and IgG2a in sera, suggesting oral tolerance induction. In *C. albicans*-inoculated mice, however, the antibody levels were the same between ovalbumin- and vehicle-fed mice. In contrast, ovalbumin feeding significantly suppressed cellular immune responses, as evidenced by reduced proliferation of splenocytes restimulated by ovalbumin *ex vivo*, in both *C. albicans*-inoculated and uninoculated mice. *Ex vivo* supplementation with neither heat-killed *C. albicans* nor the culture supernatant of *C. albicans* enhanced the production of ovalbumin-specific IgG1 in splenocytes restimulated by the antigen. These results suggest that gut colonization by *C. albicans* inhibits the induction of humoral immune tolerance to dietary antigen in mice, whereas *C. albicans* may not directly promote antibody production. We therefore propose that *C. albicans* gut colonization could be a risk factor for triggering food allergy in susceptible individuals.

Key words: *Candida albicans*; oral tolerance; ovalbumin; mice

Candida albicans is a member of the indigenous microbial flora in the gut of healthy humans [1]. However, it is also a potential pathogen and a frequent cause of complicating systemic infections and mortality in patients undergoing chemotherapy for cancer, immunosuppressive therapy or prolonged antibiotic therapy [2, 3]. In addition, it has been postulated that the excessive colonization by *C. albicans* in the gut may be responsible for a variety of hypersensitivity diseases including allergy, whereas more research is needed to understand its cause-and-effect relationships [4].

We have developed a model of sustained *C. albicans* gut colonization by a single intragastric inoculation of *C. albicans* in healthy adult mice without administration of antibiotics or immunosuppressants [5]. Although these

mice appear healthy despite lifelong *C. albicans* gut colonization under immunocompetent conditions, disseminated infection by *C. albicans* in visceral organs including the spleen, kidneys, liver, and lungs is induced upon treatment with immunosuppressive agents. Thus, these mice are useful as an animal model mimicking immunocompetent humans with chronic and latent gut colonization by *C. albicans*. Using this model, we demonstrated that serum antibody responses to repeated oral administration of ovalbumin (OVA) were enhanced by *C. albicans* gut colonization, suggesting that *C. albicans* gut colonization is likely to promote sensitization against dietary antigens and increase the risk for food allergy [6].

Exposure to soluble antigens in the gut leads to a systemic unresponsiveness to the same antigens subsequently delivered systemically, a phenomenon named oral tolerance [7, 8]. This mechanism presumably prevents the development of food allergy. Oral tolerance

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induction can be abrogated under some conditions, and this can be considered equivalent to the promotion of sensitization, which may trigger food allergy in susceptible individuals. During viral or bacterial infections in the gut, intestinal permeability to dietary antigens generally increases, because of alterations in the intestinal epithelium caused by infectious agents and by the inflammatory reaction [9]. In the context of such an inflammatory environment, local antigen-presenting cells (mainly dendritic cells) switch from a tolerogenic to an immunogenic state [10]. Indeed, some reports suggest a positive association between *Helicobacter pylori* infection and food allergy [11, 12] and atopic dermatitis [13]. Matysiak-Budnik et al. described that *H. pylori* increases the absorption of antigens across the gastric mucosa [14] and inhibits the development of oral tolerance to dietary antigens in mice [15]. Similar to *H. pylori*, therefore, gut colonization by *C. albicans* may disturb oral tolerance induction. The present study aimed to test this possibility.

MATERIALS AND METHODS

Animals

Five-week-old female specific pathogen-free BALB/c mice, which were purchased from Japan SLC (Hamamatsu, Japan), were housed in a temperature-controlled ($23 \pm 2^\circ\text{C}$) room with a dark period from 20:00 to 8:00 hr and allowed free access to sterile water and a purified diet prepared according to AIN-93G [16].

This study was approved by the Hokkaido University Animal Use Committee (approval no. 08-0139), and the animals were maintained in accordance with the guidelines for the care and use of laboratory animals of Hokkaido University.

Experimental design

After acclimatizing for two weeks, twenty-four mice were divided into two groups and then administered intragastrically either phosphate-buffered saline (PBS) containing *C. albicans* or PBS alone as described below. Weekly fecal samples were collected and cultured for *C. albicans* as described below. At three weeks after inoculation, mice in each group were further divided into two groups (six mice per group) and then administered intragastrically with either PBS containing OVA (20 mg/mouse, 5 \times crystallized, Seikagaku Corporation, Tokyo, Japan) or PBS alone for five consecutive days. At one week after completion of intragastric administration, all mice were intraperitoneally immunized with OVA (40 μg) in alum. Thereafter, blood samples were obtained from the tail vein at weekly intervals and subjected to

ELISA for measurement of OVA-specific IgG and IgE titers as described below. At three weeks after immunization, mice were anesthetized by diethyl ether and euthanized by exsanguination from the carotid artery. The blood samples were subjected to ELISA for measurement of OVA-specific IgG1 and IgG2a titers as described below. Following laparotomy, the spleen was aseptically excised and then subjected to an ex vivo antibody production assay and cell proliferation assay as described below.

Inoculation and enumeration of C. albicans

C. albicans (JCM 1542) was obtained from the Japan Collection of Microorganisms of the Institute of Physical and Chemical Research (Saitama, Japan) and maintained as previously described [5]. For inoculation, mice were acclimatized to a purified diet for two weeks before being deprived of the diet overnight. Mice were then inoculated intragastrically with 0.2 ml of PBS containing 1×10^7 cells of *C. albicans*. Uninoculated control mice were administered with 0.2 ml of PBS. Fecal specimens were quantitatively cultured using a standard pour plate technique as previously described [5].

Ex vivo experiments of splenocytes

Single-cell suspensions were prepared from spleens in RPMI 1640 (Gibco-BRL, Tokyo, Japan) supplemented with 2% heat inactivated fetal calf serum (FCS). Red blood cells were lysed with 0.83% ammonium chloride in 10 mM Tris-HCl (pH 7.4). Cell debris and clumps were removed by filtering through nylon mesh (100 μm), and cell suspensions were then made up in RPMI 1640 containing 5% heat inactivated FCS, 100 units/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin, 50 $\mu\text{g}/\text{ml}$ gentamicin, and 50 μM 2-mercaptoethanol. Cells were aliquoted at 2×10^5 cells/well in 96-well round-bottom plates along with different concentrations of OVA (0, 100 and 1,000 $\mu\text{g}/\text{ml}$). All cultures were then incubated at 37°C in a humidified 5% CO_2 atmosphere for 72 hr and pulsed with 100 μM 5-bromo-2'-deoxyuridine (BrdU) for the last 6 hr of culture. Proliferative responses were assayed using a Cell Proliferation ELISA BrdU kit (Roche, Tokyo, Japan) according to the manufacturer's recommendations. OVA-specific cell proliferation was presented as a stimulation index calculated with the following equation: stimulation index = (BrdU incorporation in the cells with OVA)/(BrdU incorporation in the cells without OVA).

For the antibody production assay, splenocytes isolated from individual mice were pooled in each group and suspended in RPMI 1640 containing 10% heat

inactivated FCS, 100 units/ml penicillin, 100 µg/ml streptomycin, 50 µg/ml gentamicin, 50 µM 2-mercaptoethanol, and 25 mM Hepes. Cells were aliquoted at 5×10^6 cells/well in 24-well plates along with or without OVA (100 µg/ml) and heat-killed *C. albicans* (10^5 cells/ml) and the supernatant of *C. albicans* culture medium prepared as described below. After 3 d of incubation at 37°C in a humidified 5% CO₂ atmosphere, cells were washed with PBS and then incubated in the medium without OVA supplementation for further 7 d. The concentration of OVA-specific IgG1 in the culture supernatant was determined by ELISA as described below. The culture was performed in triplicate.

Preparation of C. albicans culture supernatant and heat-killed C. albicans

C. albicans was maintained as described above. Cells grown in the yeast form were suspended in PBS and then incubated at 37°C for 2.5 hr. The culture supernatant was obtained by centrifugation at $3,000 \times g$ for 10 min, and the cell pellet was suspended in PBS and autoclaved at 121°C for 0.5 hr. Because *C. albicans* shows hyphal growth when it colonizes in the gut mucosa [5], the culture supernatant and heat-killed cells were also prepared from hyphally grown *C. albicans*. Hyphal growth was induced by culturing the yeast cells (10^6 cells/ml) of *C. albicans* in PBS supplemented with 10% FCS at 37°C for 2.5 hr. The culture supernatant and heat-killed cells were obtained as described above.

ELISA for anti-OVA antibodies

OVA-specific antibodies in the culture supernatant of splenocytes and serum were determined by ELISA as previously described [6]. In brief, 96-well microtiter plates were coated with 200 µg/ml OVA, and culture supernatant and serially diluted sera were then added and incubated. For OVA-specific IgG, IgG1 and IgG2a, HRP-conjugated goat anti-mouse IgG, IgG1 and IgG2a (Zymed, San Francisco, CA, USA) diluted 1:2,000, 1:1,000 and 1:1,000, respectively, were added and incubated. Plates were developed after addition of o-phenylenediamine (0.4 mg/ml) and hydrogen peroxide (0.012%). Finally, 1 M H₂SO₄ was added, and absorbance was measured at 490 nm. For OVA-specific IgE, 96-well microtiter plates were coated with 10 µg/ml anti-mouse IgE monoclonal antibody (LO-ME-2, Zymed), and serially diluted sera were then added and incubated. After the incubation, OVA-DIG conjugate (20 ng/ml) was added and incubated. Coupling of DIG to OVA was performed using a DIG Protein Labeling Kit (Roche) according to the manufacturer's instructions.

HRP-conjugated sheep anti-DIG Fab fragments (Roche) diluted 1:4,000 were then added and incubated. Plates were developed at room temperature after addition of 3,3',5,5'-tetramethylbenzidine (0.123 mg/ml, Sigma, St. Louis, MO, USA) and hydrogen peroxide (0.012%). Finally, 1 M H₂SO₄ was added, and the absorbance was measured at 450 nm. Pre-immunized serum was used as a negative control. The average absorbance in the negative control wells, to which three times the standard deviation was added, provided the reference for determination of the titer in the test sera. Antibody titers were expressed as the reciprocal of the last dilution yielding an extinction value higher than the reference value. For OVA-specific IgG1 in the culture supernatant of splenocytes, the absorbance was shown as an indicator of antibody concentrations.

STATISTICS

Results are presented as means \pm SEM. The Tukey-Kramer test following one-way or two-way ANOVA was used to compare mean values. GraphPad Prism for Macintosh (version 5.0, GraphPad Software, Inc., La Jolla, CA, USA) was used for the analysis.

RESULTS

To study the effect of chronic gut colonization by *C. albicans* on oral tolerance induction, BALB/c mice were intragastrically inoculated with *C. albicans*. By weekly enumeration in fecal specimens after inoculation, a high fecal recovery of *C. albicans* was observed in all mice throughout the experimental period (approx. 1×10^7 colony forming units/g feces). In addition, enumeration of organisms in gut tissues by quantitative culture after euthanasia of animals revealed that the colonization occurred in the stomach, small intestine, and large intestine in all mice (data not shown). No organism was detected in the feces and tissues of uninoculated control mice. All mice showed a good health appearance. These results indicate that chronic asymptomatic colonization by *C. albicans* was established in the gut of mice after a single inoculation of *C. albicans*.

Figure 1 shows the time course of changes in OVA-specific antibody titers in sera after intraperitoneal immunization. OVA-specific IgG and IgE began increasing at one week after immunization (Fig. 1A and 1B, respectively). At two and three weeks after immunization, the OVA-specific IgG and IgE were significantly lower in those fed OVA than in those fed PBS. In *C. albicans*-inoculated mice, however, there were no significant differences in OVA-specific IgG and IgE titers between OVA-fed and PBS-fed mice. In PBS-

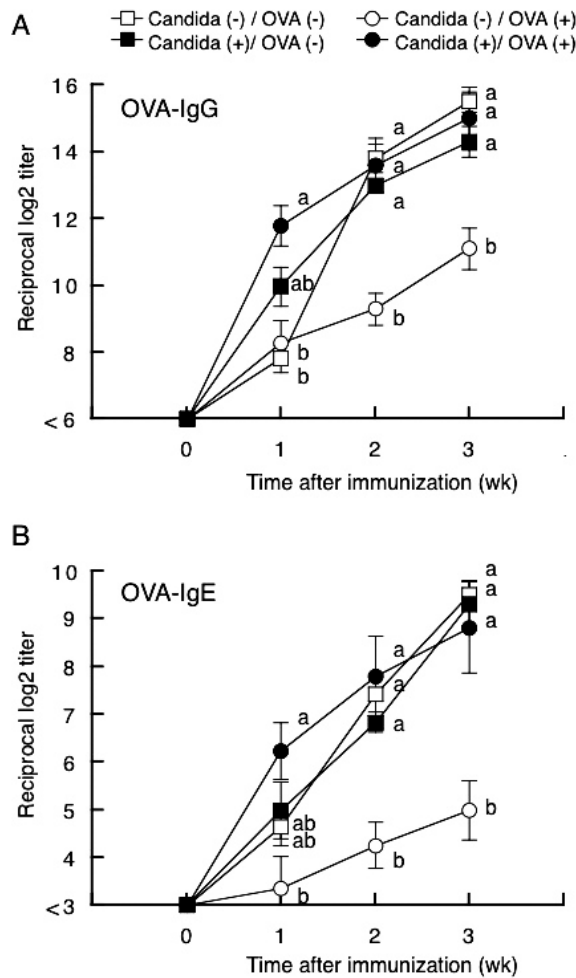


Fig. 1. Time course of changes in OVA-specific IgG (panel A) and IgE (panel B) titers in sera of BALB/c mice with (closed symbols) or without (open symbols) *C. albicans* gut colonization. Mice fed OVA (closed and open circles) or PBS (closed and open triangles) were intraperitoneally immunized with OVA in alum, and weekly blood samples were then subjected to ELISA. Values are presented as means \pm SEM of six mice per group. Values not sharing the same letters at each time point are significantly different at $p < 0.05$.

fed mice, there were no significant differences in OVA-specific IgG and IgE titers between *C. albicans*-inoculated and uninoculated mice.

OVA-specific IgG1 and IgG2a (Fig. 2A and 2B, respectively) titers were determined in sera obtained on the last day of the experiment. In uninoculated mice, IgG1 and IgG2a titers were significantly lower in mice fed OVA than in mice fed PBS, whereas there was no significant difference between OVA-fed and PBS-fed mice in the *C. albicans*-inoculated group. In mice fed PBS, there were no significant differences in OVA-

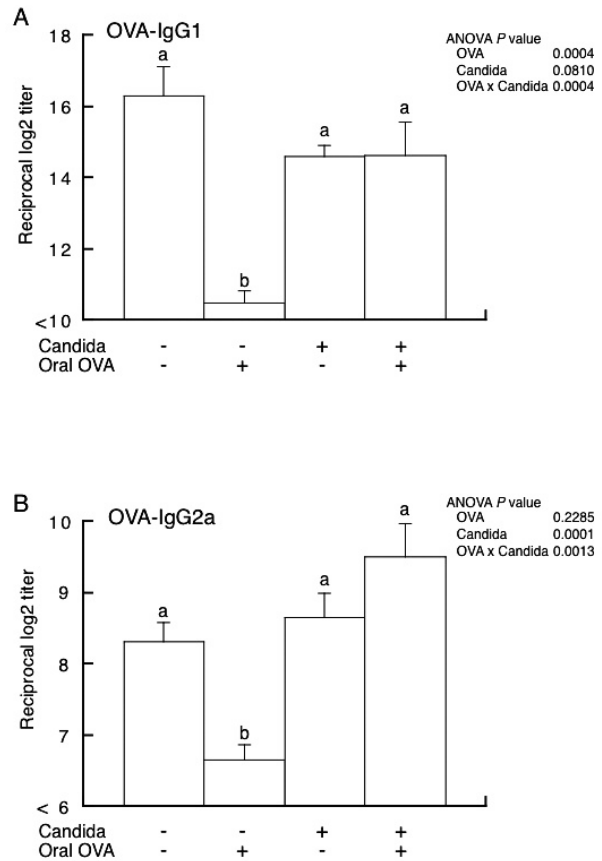


Fig. 2. OVA-specific IgG1 (panel A) and IgG2a (panel B) titers in sera of BALB/c mice with or without *C. albicans* gut colonization. Mice fed OVA (OVA (+)) or PBS (OVA (-)) were intraperitoneally immunized with OVA in alum. Blood samples three weeks after immunization were subjected to ELISA. Values are presented as means \pm SEM of six mice per group. p values for two-way ANOVA are shown. Values not sharing the same letters are significantly different at $p < 0.05$ as estimated by a post-hoc Tukey-Kramer test.

specific IgG1 and IgG2a titers between *C. albicans*-inoculated and uninoculated mice.

Oral tolerance induction was also examined ex vivo by assessing antigen-specific proliferation of splenocytes. OVA feeding significantly reduced the OVA-specific cell proliferation measured by BrdU incorporation in both *C. albicans*-inoculated and uninoculated mice (Fig. 3). *C. albicans*-inoculation had no influence on cell proliferation.

In our preliminary experiments, ex vivo stimulation by OVA induced production of OVA-specific IgG1 but not IgE and IgG2a in splenocytes under our experimental conditions. Therefore, we measured OVA-specific IgG1 in order to test whether ex vivo supplementation with *C. albicans* promotes antibody production in splenocytes.

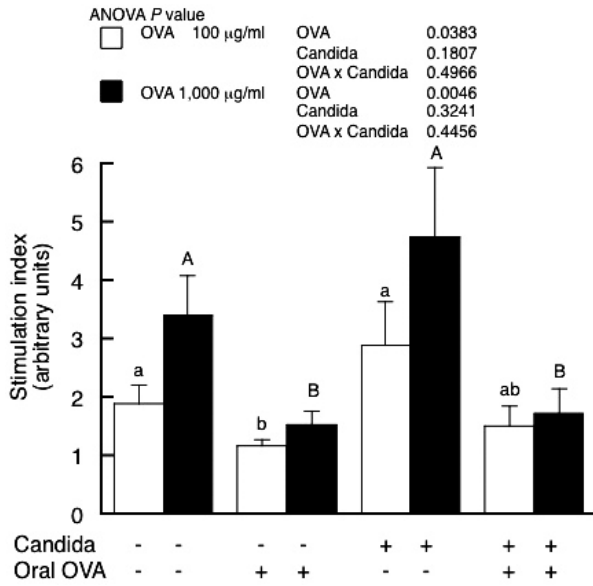


Fig. 3. OVA-specific splenocyte proliferation in BALB/c mice with or without *C. albicans* gut colonization. Mice were fed either OVA (OVA (+)) or PBS (OVA (-)) and then intraperitoneally immunized with OVA in alum. The spleen was removed three weeks after immunization, and splenocytes were then cultured with 100 and 1,000 µg/ml OVA (open and closed columns, respectively). Cell proliferation was assessed by BrdU incorporation and presented as a stimulation index as described in the Materials and Methods section. Values are presented as means ± SEM of six mice per group. p values for two-way ANOVA are shown. Values not sharing the same letters are significantly different at p < 0.05 as estimated by a post-hoc Tukey-Kramer test.

In the splenocytes isolated from *C. albicans*-uninoculated mice, ex vivo stimulation by OVA promoted production of OVA-specific IgG1 in PBS-fed mice but not in OVA-fed mice (Fig. 4A). In contrast, OVA-specific IgG1 production was increased by OVA stimulation in the splenocytes isolated from *C. albicans*-inoculated and OVA-fed mice. However, ex vivo supplementation with neither heat-killed *C. albicans* nor the culture supernatant of *C. albicans* grown in the yeast and hyphal form increased the IgG1 production in the splenocytes isolated from uninoculated and OVA-fed mice (Fig. 4B). In fact, ex vivo supplementation with *C. albicans* tended to reduce the IgG1 production in the splenocytes isolated from PBS-fed mice.

DISCUSSION

The present study investigated the development of oral immune tolerance to dietary antigens in a mouse model for chronic and asymptomatic colonization by *C. albicans* in the gut. Our data clearly showed that *C.*

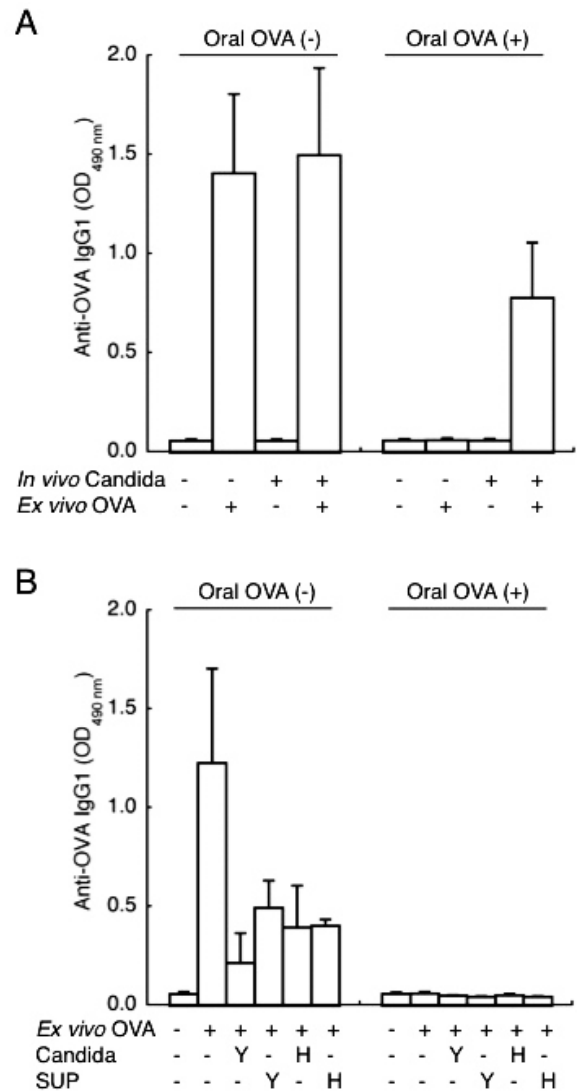


Fig. 4. OVA-specific IgG1 levels in the culture supernatant of splenocytes isolated from BALB/c mice with or without *C. albicans* gut colonization. Mice fed OVA (Oral OVA (+)) or PBS (Oral OVA (-)) were intraperitoneally immunized with OVA in alum. The spleen was removed three weeks after immunization, and splenocytes isolated from individual mice were pooled in each group and then subjected to an ex vivo antibody production assay. Panel A shows the effect of ex vivo stimulation by OVA (100 µg/ml), and panel B shows the effect of ex vivo supplementation with heat-killed *C. albicans* (Candida) and the culture supernatant of *C. albicans* (SUP) prepared in the yeast (Y) and hyphal (H) forms. Values are presented as means ± SEM of three wells.

albicans gut colonization inhibits antigen feeding-induced suppression of serum antibody responses to systemic immunization, suggesting the inhibition of oral

tolerance induction by *C. albicans* gut colonization. We previously reported that *C. albicans* gut colonization promotes serum IgE and IgG antibody responses to repeated oral administration with OVA in mice [6]. Thus, the inhibition of oral tolerance induction appears to explain the increased responses of serum antibodies to repeated oral antigen exposure in *C. albicans*-colonized mice.

Gastrointestinal pathogens and their products have been reported to disrupt oral tolerance induction. Cholera toxin has been well known as a mucosal adjuvant that abrogates oral tolerance to an unrelated protein fed simultaneously in mice [17]. In addition, *Escherichia coli*-heat labile enterotoxin has been reported to disrupt oral tolerance induction in mice [18]. Furthermore, gastric infection with *H. pylori* reportedly inhibited the development of oral tolerance by preventing anti-OVA IgE suppression in mice [15]. Because oral tolerance to dietary antigens presumably prevents the development of food allergy, these findings suggest that gastrointestinal infections could trigger food allergy in susceptible individuals. Indeed, *H. pylori* infection has been reported to have a positive association with the development of food allergy [11, 12]. Therefore, the present study suggests that *C. albicans* colonized in the gut could be a pathogen triggering food allergy through disrupted induction of oral tolerance by preventing dietary-antigen specific antibody suppression.

Cellular and molecular mechanisms for disrupted induction of oral tolerance in *C. albicans*-colonized mice remain to be elucidated. CD4⁺ effector T cells can be classified as Th1 and Th2 and, also newly identified Th17 cells, according to their profile of cytokine secretion [19, 20]. Because Th1 and Th2 cytokines are responsible for cellular and humoral immune responses, respectively, one may suspect that disruption of oral tolerance induction by preventing antibody suppression is associated with a Th2-skewed condition in *C. albicans*-colonized mice. In mice, IgG antibodies are classified as Th1-dependent isotype (IgG2a) and Th2-dependent isotypes (IgG1). In the present study, *C. albicans* colonization inhibited suppression of both IgG2a and IgG1 antibodies in OVA-fed mice. It is therefore unlikely that the inhibition of antibody suppression by *C. albicans* colonization is due to a Th2-skewed response, although additional studies are needed to show the response of Th1 and Th2 cytokines (e.g., IFN- γ and IL-4, respectively). Alternatively, *C. albicans* colonization might affect the induction of regulatory T cells (Treg) because Treg cells are involved in the oral tolerance induction by suppressing both Th1 and Th2

responses [21]. We are now investigating whether *C. albicans* gut colonization influences the responses of Th1, Th2, Th17 and Treg cells under an experimental setting in which oral tolerance is induced.

In the present study, because antigen feeding-induced suppression of splenocyte proliferation was still observed in *C. albicans*-colonized mice, it appears that the reduced response of cellular immunity in antigen-fed mice is unaffected by *C. albicans* colonization. Therefore, one may speculate that *C. albicans* might stimulate plasma cells to produce antibodies. The present study tested whether *C. albicans* stimulates antibody production in splenocytes ex vivo. In the splenocytes isolated from *C. albicans*-uninoculated mice, ex vivo stimulation by OVA promoted production of OVA-specific IgG1 in PBS-fed mice, whereas IgG1 production was not promoted by ex vivo OVA in OVA-fed mice. In contrast, in *C. albicans*-inoculated mice, ex vivo stimulation by OVA promoted IgG1 production in the splenocytes isolated from both PBS- and OVA-fed mice. These results suggest that antibody production in splenocytes restimulated by an antigen ex vivo is inhibited by antigen feeding prior to immunization, and that *C. albicans* gut colonization inhibits the antigen feeding-induced suppression of antibody production in splenocytes. Thus, these ex vivo observation appear to reflect the *in vivo* findings. Under these conditions, supplementation with neither heat-killed *C. albicans* nor the culture supernatant of *C. albicans* promoted production of OVA-specific IgG1 in the splenocytes isolated from OVA-fed mice. In addition, in the splenocytes isolated from PBS-fed mice, IgG1 production was rather reduced by supplementation with both heat-killed *C. albicans* and the culture supernatant of *C. albicans*. To understand the mechanism for these phenomena, we are now investigating cytokine production in splenocytes ex vivo. Based on the above, it is unlikely that the cell constituents of *C. albicans* directly promote antibody production in splenocytes restimulated by an antigen and abrogate already established oral tolerance in splenocytes. Although Tokunaka et al. reported that intraperitoneal administration of β -D-glucan, a cell-wall constituent of *C. albicans*, along with bovine serum albumin (BSA) promoted an increase in serum anti-BSA IgG in mice [22], our previous study showed that the serum concentrations of β -D-glucan in *C. albicans*-colonized mice did not differ from those of the control mice [23]. In our model of *C. albicans*-colonized mice, therefore, it is unlikely that β -D-glucan released from *C. albicans* is absorbed in the gut and then contributes to the inhibition

of antigen feeding-induced suppression of serum antibodies.

Similar to the present study, Noverr et al. previously described the effect of *C. albicans* gut colonization on the induction of immune tolerance in mice [24]. The authors observed that repeated intranasal administration with OVA in antibiotics-administered and *C. albicans*-colonized BALB/c mice without previous systemic immunization resulted in an allergic airway response characterized by increased eosinophils and Th2 cytokines in the lung and increased serum IgE. Because repeated airway exposure to an antigen usually leads to tolerance rather than sensitization [25–27], the findings by Noverr et al. suggest disrupted induction of airway mucosal tolerance in *C. albicans*-colonized mice. In addition, Noverr et al. reported that *C. albicans* secretes prostaglandin-like oxylipin molecules having immunomodulatory actions [28, 29]. These molecules might be involved in the inhibition of oral tolerance development by *C. albicans* gut colonization.

Pattern recognition receptors (PRRs), such as C-type lectin receptors (CLRs) and Toll-like receptors (TLRs), are expressed in the host cells and sense pathogen-associated molecular patterns (PAMPs) including β -glucans, chitin, and mannans in *C. albicans* [30–33]. CLRs such as dectin-1 are central to the recognition of PAMPs in *C. albicans*, and TLR2, TLR4 and TLR9 are also involved in sensing the PAMPs. The recognition of PAMPs by PRRs on phagocytes contributes to the antifungal innate immune response through phagocytosis and direct pathogen killing, while uptake of *C. albicans* by dendritic cells (DCs) promotes the adaptive immune response, i.e., the differentiation of naïve T cells into effector T cell subtypes. Specifically, inflammatory DCs initiate antifungal Th2 and Th17 cell responses, whereas tolerogenic DCs activate Th1 and Treg cell differentiation [34]. Thus, the immune response eliminates the fungus while preventing excessive inflammation. Indeed, *C. albicans* and the fungal product zymosan reportedly activated a tolerogenic program in the gut, resulting in the activation of Treg cell-dependent immune tolerance [34–36]. These findings are in contrast to those of the present study, but whether PAMPs in *C. albicans* promote the induction of humoral immune tolerance by dietary antigens remains to be elucidated.

In conclusion, the present results suggest that *C. albicans* gut colonization inhibits oral tolerance induction by preventing antibody suppression in mice. The disrupted induction of oral tolerance would lead to sensitization to repeated antigen exposure in the gut of *C. albicans*-colonized mice. We therefore propose that *C.*

albicans gut colonization could be a risk factor for triggering food allergy in susceptible individuals.

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