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Oral administration of whole dihomo- γ -linolenic acid-producing yeast suppresses allergic contact dermatitis in mice

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ABSTRACT

Dihomo- γ -linolenic acid (DGLA, C20: 3n-6) is known to have an anti-inflammatory activity, but its range of effects was not well studied because of its limited natural sources. We addressed these issues by constructing a yeast *Saccharomyces cerevisiae* strain having a complete metabolic pathway for DGLA synthesis by introducing two desaturase and one elongase genes to convert endogenous oleic acid to DGLA. Taking advantage of well-known safety of *S. cerevisiae*, we previously investigated the efficacy of heat-killed whole DGLA-producing yeast cells on irritant contact dermatitis, and showed that oral intake of this yeast significantly suppressed inflammatory reactions, whereas no such suppression was observed by the intake of 25 times the amount of purified DGLA. Since this method is considered to be a simple and efficient way to suppress inflammation, we examined its effectiveness against allergic contact dermatitis (ACD) in this study and showed that this method was also effective against ACD.

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The polyunsaturated fatty acids (PUFAs) have been attracting considerable interest because of their important roles in human health and nutrition. For instance, arachidonic acid (ARA, C20:4n-6) and docosahexaenoic acid (DHA, C22:6n-3) are essential fatty acids found in brain tissues. They are also important dietary nutrients for neonatal babies owing to their involvement in the development of neural and retinal functions [1–5]. Fatty acids (FAs) in phospholipids contribute to determine the characteristics of various lipid bilayer membranes. At the same time, PUFAs are converted to eicosanoids, and those like prostaglandins, leukotrienes, and thromboxanes mediate fever, inflammations, vasodilatation, blood pressure, clotting, pain, neurotransmission, and modulation of cholesterol metabolism[6].

The principal sources for PUFAs (especially n-3 fatty acids) to date are fish oils. However, due to the increase of fish consumption and the expansion of marine pollution, fish hauls are decreasing. It is therefore highly desirable to produce PUFAs from alternative sources that are more economical, easier to handle, and sustainable through genetic engineering techniques. One option is to improve oil-seed crops to produce PUFAs through genetic engineering technique, and the other option is to produce them in well-studied model microorganisms such as *S. cerevisiae*. Since *S. cerevisiae* has been serving as a model organism for the development of metabolic engineering strategies to produce various metabolites [7], we took the latter option and constructed

a complete pathway for dihomo- γ -linolenic acid (DGLA) synthesis in *S. cerevisiae*, which is originally able of produce only monounsaturated palmitoleic (C16:1n-7) and oleic (C18:1n-9) acids [8] as it contains only $\Delta 9$ -fatty acid desaturase gene (*OLE1*). Thus, we made it possible to convert endogenous oleic acid to DGLA by introducing *Kluyveromyces lactis* $\Delta 12$ fatty acid desaturase (*KIFAD2*), rat $\Delta 6$ -fatty acid desaturase (*rFADS2*), and rat elongase (*rELO1*) genes (Figure 1) [9]. Production of DGLA in this reconstituted yeast was not so high, but by taking advantage of its traditionally established safety in relation to fermented foods and beverages, we administered heat-killed whole DGLA-producing yeast cells instead of purified lipids or fatty acids to mice, and showed that oral intake of this yeast, but not the GLA-producing yeast nor the control empty-vector yeast, significantly suppressed irritant contact dermatitis (ICD) reactions induced by topical application of croton oil to mouse ears[10]. The effect was observed by only 7-day advanced administration of DGLA as a form of yeast, but 25 times the amount of purified DGLA did not work, suggesting this method is simple but very efficient to suppress inflammatory responses. Therefore, to extend its application, we examined in this study its effectiveness against allergic contact dermatitis (ACD), also known as common skin disease like ICD. In contrast to ICD, ACD is a T cell-mediated type IV delayed hypersensitivity reaction. Exposure to an antigen (sensitization) and subsequent challenge with the same antigen (elicitation)

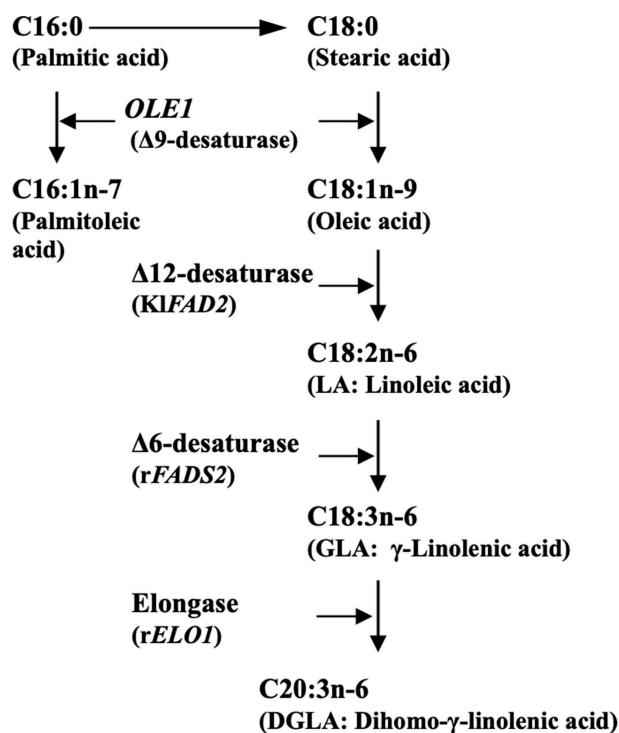


Figure 1. Biosynthetic route for DGLA production.

YHU3046-4A strain expressing *KIFAD2*, *rFADS2*, and *rELO1* genes was used as a DGLA-producing yeast strain, which contains approximately 2.4 μg and 3.0 μg of GLA and DGLA per mg of dry cell weight, respectively. DGLA-producing yeast cells equivalent to 50 mL and 100 mL are referred to as DGLA and 2 x DGLA, respectively.

causes the recruitment and activation of hapten-specific T cells at the challenged skin sites followed by the infiltration of hapten-nonspecific leukocytes, including neutrophils and monocytes. As a result, ACD reactions characterized by erythema and edema peak within 24 to 48 h after challenge[11]. We demonstrated that oral intake of the DGLA-producing yeast was also effective against ACD although almost double amount of yeast cells were required to suppress inflammatory responses.

Materials and methods

S. cerevisiae strain, media, and growth

S. cerevisiae strain YHU3046-4A (*MATa leu2-3 leu2-112 ura3-52 his3::KanMX3*) harboring pL2308-7 (*KIFAD2* expressed under the control of the *ADH1* promoter on a *LEU2* marker plasmid pL1177-2), pL2314-136 [rat Δ6 fatty acid desaturase (*rFADS2*) expressed under the control of the *TDH3* promoter on a *URA3* marker plasmid YEp352-GAP], and pL2308-8 [rat elongase (*rELO1*) expressed under the control of the *ADH1* promoter on a *HIS3* marker plasmid pRS13] was used as a DGLA-producing strain[9].

The DGLA-producing strain was grown as described previously[10]. Briefly, the strain was pre-cultured in the NSD medium at 30°C twice to increase culture volume stepwise. Then, the resultant

cultures were grown at 15°C for 7 days to increase the yield of DGLA[9]. After cultivation, cells were harvested by centrifugation, suspended in one-fortieth volume of distilled water and autoclaved. The autoclaved yeast cells were divided into small tubes for daily oral administration and cell pellets were kept at −80°C until use.

Total fatty acid analysis

Total fatty acid contents in the autoclaved yeast cells were determined by gas chromatographic analysis. Fatty acid analysis was performed by the application of 0.2 μL aliquots to a gas chromatograph (GC2010, Shimadzu, Kyoto, Japan) equipped with a TC-70 capillary column (30 m x 0.25 mm i.d., GL Sciences, Tokyo, Japan) under the temperature programming (160–234°C at 4.5°C/min increments). The fatty acid composition was calculated based on the area of each peak, and the amount was determined by comparison with the methylheptadecanoate (C17:0) standard.

Reagents

Commercially available DGLA was purchased from Cayman Chemical Co. (Ann Arbor, MI, USA). Dinitrofluorobenzene (DNFB) was purchased from Sigma (St. Louis, MO, USA). For its application on mice, 25 μL of DNFB was dissolved in 4 mL of acetone and 1 mL of olive oil to make its final concentration of 0.5%.

Animals and feeding conditions

Five-week-old female ddY mice were purchased from Sankyo Labo Service Co. (Tokyo, Japan). Mice were kept under controlled temperature (25 °C) and humidity (40 ± 5%) on a 12 h dark – 12 h light cycle at the Animal Research Center of Toho University, Narashino Campus. They were given a commercial diet (Labo MR stock, Nossan corporation Life-Tech Department, Kanagawa, Japan) and water *ad libitum*. Yeast cells and purified DGLA were orally administered once a day. Yeast cells were administered as pellets. Fifty microliter of purified DGLA solutions with various dilutions in ethanol (EtOH) were mixed with 100 μL of corn oil as a carrier and administered to mice. Mice were used in accordance with the Animal Experimental Guidelines for Toho University Animal Care and User Committee.

Induction of ACD

As shown in Figure 2(a), yeast cells were orally supplied to mice once a day for 10 days. On day 2, 100 μL of 0.5% DNFB solution was applied on the

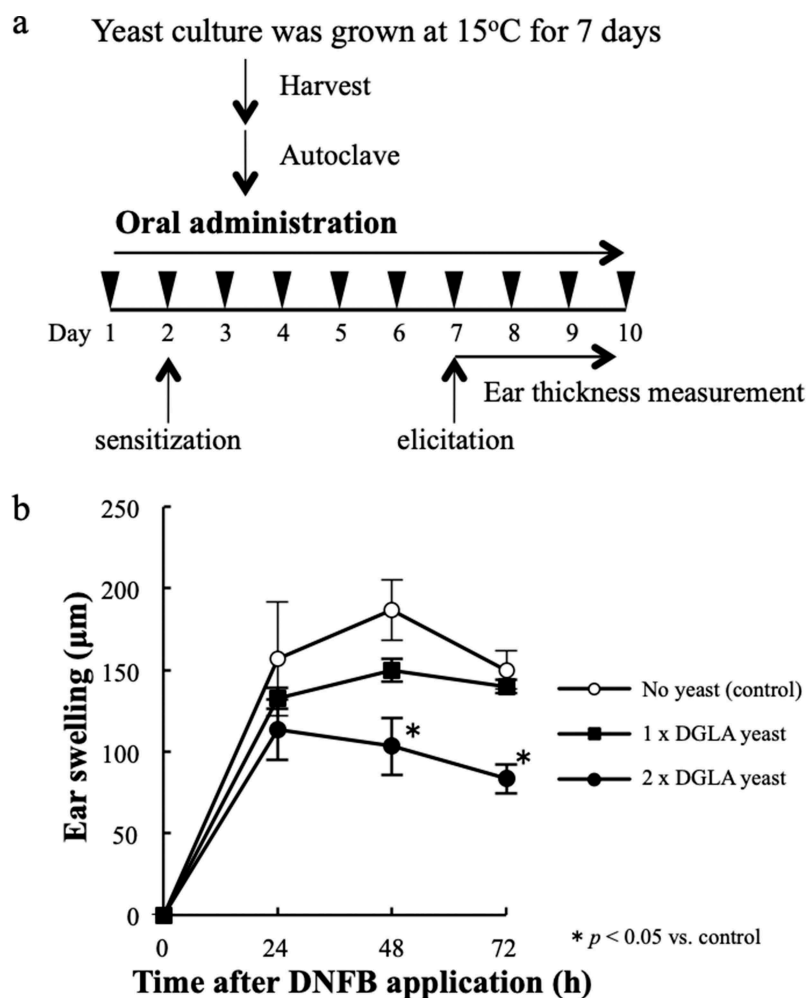


Figure 2. Dose-dependent suppression of ACD by oral intake of DGLA-producing yeast cells.

(a) The schematic diagram for sensitization and elicitation of mice by DNFB. Mice were orally administered with DGLA-producing yeast cells containing around 0.22 mg of DGLA (1 x DGLA) or 0.44 mg of DGLA (2 x DGLA) once a day for 10 days. After sensitization on day 2 and the following elicitation by DNFB on day 7, ear thickness was measured for 3 days with a continuous feeding of the DGLA-producing yeast. As a control of DNFB, the same volume of acetone and olive oil mixture was applied to left ears as a control. (b) Time course of ear swelling in no yeast (open circles), 1 x DGLA yeast (closed squares), and 2 x DGLA yeast (closed circles)-supplied mice is shown. Results are expressed as the means $\pm$ SE of 4 mice. * $p < 0.05$ versus no yeast control by one-way ANOVA with Dunnett's test.

shaved back skin of mice for sensitization. Five days later, 25 μ L of 0.5% DNFB solution was applied to the right ears of mice for elicitation. The same volume of acetone and olive oil mixture was applied to left ears as a control. Time course of ear swelling was monitored by measuring ear thickness using a dial thickness gauge (Ozaki Manufacturing Co., Tokyo, Japan) for the following 3 days. Usually, four mice were used for one experiment.

Histological analysis

Mouse ear tissues fixed with 10% formaldehyde were embedded in paraffin and kept stored at room temperature until processed for microscopic observation. Five micrometer-thick sections were prepared at a later date, stained with hematoxylin and eosin, and photographed under the light microscope to examine histological lesions.

Enzyme-linked immunosorbent assay (ELISA)

Cell lysates from ear tissues were prepared as described, and concentrations of keratinocyte-derived (KC) and monocyte chemoattractant protein-1 (MCP-1) chemokines in the cell lysates were measured by the solid-phase ELISA kit from PeproTech (Rocky Hill, NJ, USA) using the protocols recommended by the manufacturer[10]. Likewise, the concentration of interferon- γ (IFN- γ) cytokine was measured by the solid-phase ELISA kit from BioLegend (San Diego, CA, USA). Their concentrations were expressed as ng/mg protein of cell lysate.

Statistical analysis

All values are expressed as means $\pm$ SE. Statistical differences were examined by Dunnett's test. P values < 0.05 were considered statistically significant.

Results

Suppression of ACD by oral intake of DGLA-producing yeast cells

We chose DGLA among beneficial PUFAs because its range of anti-inflammatory activities was not well-understood due to limited natural sources [12,13], and we reconstituted a complete pathway to produce DGLA in *S. cerevisiae* by introducing two types of desaturase and one elongase genes (Figure 1). Although the production of DGLA in this reconstituted yeast strain was not so high, we showed in the previous study that oral intake of the whole DGLA-producing yeast cells was a very efficient method to suppress ICD induced by a topical application of croton oil to mouse ears[10].

To extend the application of this method, we examined its effectiveness against ACD in this study. Mice were orally administered with DGLA-producing yeast cells equivalent to 50 mL and 100 mL of culture containing approximately 0.22 mg and 0.44 mg of DGLA, respectively, once a day for 10 days (hereafter referred to as 1 x DGLA and 2 x DGLA, respectively, see Figure 2(b)). The amount of yeast corresponds to DGLA was the amount that efficiently suppressed ICD in our previous study[10]. DNFB was applied to the ears of mice for elicitation on day 7 right after the administration of the DGLA-producing yeast, and ear thickness was measured for the following 3 days (Figure 2(a)). Ears of control mice with no yeast administration became swollen more than 150 μ m having the biggest impact at around 48 h. In contrast, significant suppression of ear swelling was observed by feeding of 2 x DGLA yeast for 7 days, whereas no suppression was observed by feeding of 1 x DGLA yeast (Figure 2(b)), indicating a larger amount of DGLA was required to suppress ACD caused by DNFB. Since a longer administration of 2 x DGLA for 14 days (sensitization on day 9 and elicitation on day 14) did not further enhance the degree of suppression (data not shown), administration period before elicitation was fixed to 7 days from now on.

Histological and biochemical analyses of DGLA-yeast-treated mice

Figure 3 shows histological changes in ear edema after oral intake of the DGLA-producing yeast for 7 days. Along with inflammation, ear swelling and infiltration of neutrophils (dark stained cells with characteristic morphology) into sites of inflammation were observed in the ears of control mice with no yeast administration. Typical neutrophils are indicated by arrowheads in Figure 3(d,f). On the other hand, significant reduction of ear swelling and infiltrating cells was observed in the ears of 2 x DGLA treated mice (compare Figure 3(c,d) to Figure 3(e,f)).

To further confirm the suppression of inflammation biochemically, levels of IFN γ cytokine, which is produced from T cells at the early stage of ACD, and the following induction of KC and MCP-1 chemokines were measured using ELISA kits. Their levels were reduced by DGLA treatment (Figure 4).

Effect of oral intake of purely chemical DGLA on ACD

To find the advantage of whole yeast cells over the purified lipids or fatty acids, we compared the effect of purified DGLA with that of the DGLA-producing yeast. Yeast treatment of 2 x DGLA provides approximately 0.4 mg of DGLA a day. Thus, a comparable amount of purified DGLA (0.4 mg) and 10 times DGLA (4 mg) were prepared in 50 μ L of EtOH. Since the amount was small, DGLA solutions were mixed with 100 μ L of corn oil and administrated to mice. After 7-day administration, DNFB was applied to the ear of mice and ear thickness was measured. In spite of the administration of 10-times the amount of purified DGLA, it did not suppress the inflammation at all (Figure 5), indicating administration of DGLA-producing yeast cells worked more efficiently compared to that of the purified DGLA as we have shown in our previous study on ICD[10].

Discussion

Since the application of whole yeast cells instead of purified DGLA was very efficient and required a smaller amount of DGLA compared to that of a purified one, we investigated in this study whether this method is applicable not only to ICD but also to ACD. A series of inflammatory reactions including ear swelling, accumulation of infiltrating cells into the skin sites, and increased production of cytokines and chemokines were significantly suppressed by the oral intake of DGLA-producing yeast for only 10 days, although the effective amount of DGLA-producing yeast was double compared to the previous ICD experiments. Because IFN- γ is critical for the development of DNFB-induced ACD[14], the reduction of IFN- γ might be involved in the suppression of ACD in DGLA-producing yeast-fed mice. Since DGLA inhibits proliferation of T cells[15], a major producer of IFN- γ , it is possible that the decreased IFN- γ was caused by the reduction of T cell number in DGLA-producing yeast-fed mice. In addition to IFN- γ , KC, a neutrophil-recruiting chemokine, and MCP-1, a chemotactic cytokine for monocytes, also play important roles in ACD, because treatment with antibodies to KC or MCP-1 leads to reduce ACD induced by DNFB [16,17]. Thus, decreased KC and MCP-1 production by the administration of DGLA-producing yeast may

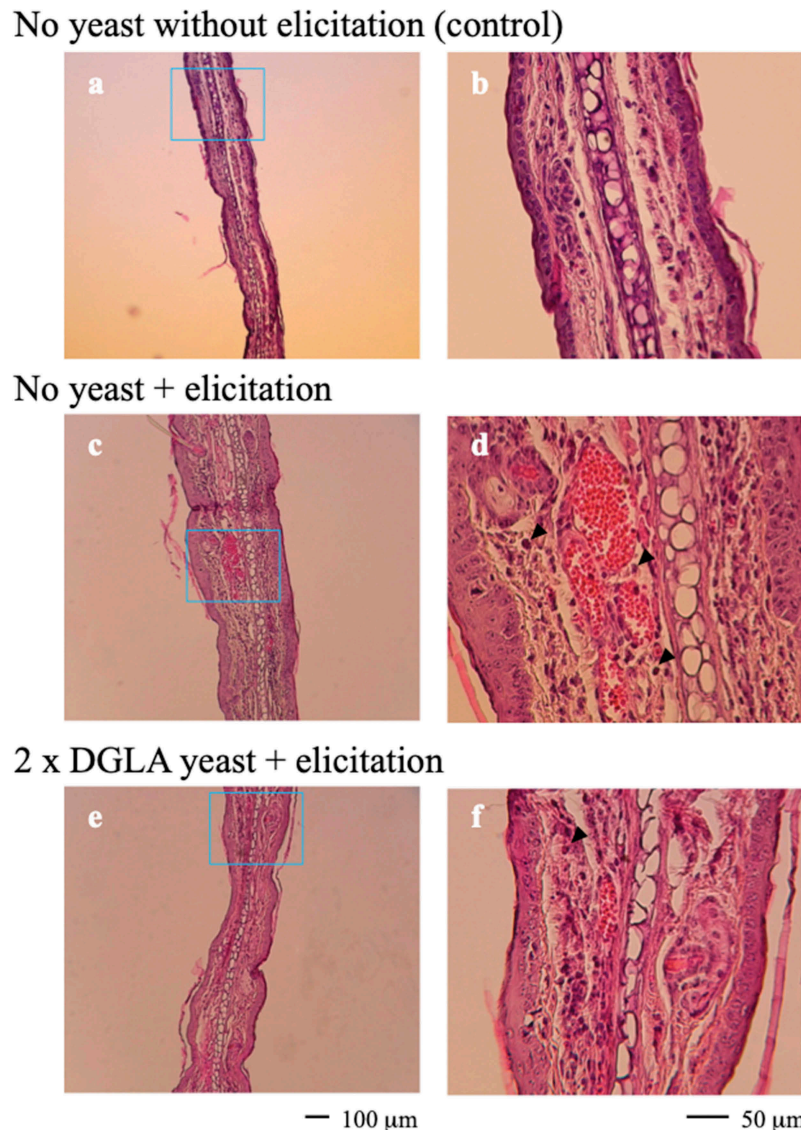


Figure 3. Histological analysis of DNFB-induced ear skin.

Mice were orally administered with DGLA-producing yeast cells (2 x DGLA) once a day for 7 days, and then DNFB was applied to their ears. Ear sections of uninduced control mice (no yeast supplied, a and b), DNFB induced (72 h) control mice (c and d), and DNFB induced (72 h) mice with 2 x DGLA-supplied (e and f) were stained with hematoxylin and eosin. Central narrow lines indicate cartilages and infiltration of inflammatory cells are observed as dark stained cells with hematoxylin. Squares indicate areas used for magnification. Typical dark-stained neutrophils with characteristic morphology are indicated by arrowheads.

contribute to suppression of ACD. Further studies for the mechanism of DGLA-induced ACD suppression are required. Similar to the previous experiments, 10 times the amount of purified DGLA did not suppress ACD, confirming its simplicity and efficacy on ACD as well as ICD although their original induction mechanisms are different. Kawashima et al. [18] reported that oral administration of DGLA enriched oil purified from *Mortierella alpina* prevented the development of atopic dermatitis in NC/Nga mice by giving a pellet diet containing 0.5 g DGLA/kg diet ad libitum for 8 weeks. It was estimated that one mouse with this diet took approximately 2 mg DGLA a day in their experiments, based on our measurement that one mouse eats around 4 g of diet a day on average. Since the experimental systems such as strains of mice, observed inflammation, and way of feeding were different between their

experiments and ours, we cannot compare these experiments directly. But it seems that their experiments also support the advantage of our delivery system to provide whole DGLA-producing yeast cells, which do not require extraction and purification of lipids/fatty acids from yeast cells, in both amount and duration of administration.

It remains to be seen how the whole yeast cells showed the advantages described above. We think yeast cells might function like capsules to protect a small amount of DGLA in cells and to prevent a loss of administration. Davis et al. [19] demonstrated the enhanced efficacy of oral delivery of ascorbic acid by its encapsulation in a liposome, and suggested its accelerated intestinal absorption, increased stability, and hence greater bioavailability as possible advantages of encapsulation of ascorbic

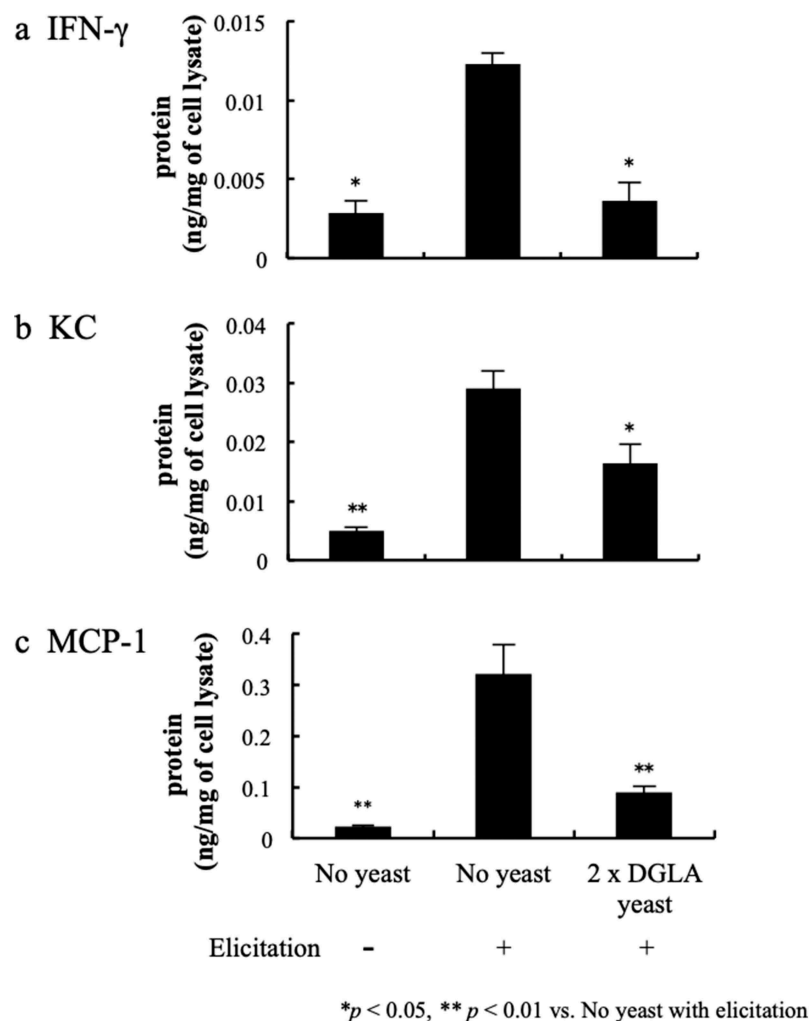


Figure 4. Effect of DGLA on cytokine and chemokine production induced by cutaneous inflammation.

Cell lysates were prepared from the ears of 48 h after DNFB application in control (No yeast with elicitation) and 2 x DGLA-supplied mice or those in uninduced control mice without yeast administration (No yeast without elicitation). Levels of IFN- γ (a), KC (b), and MCP-1 (c) were measured using ELISA kits. Results are expressed as ng/mg ear protein of cell lysate and shown as the means $\pm$ SE of triplicate determinations. * $p < 0.05$, ** $p < 0.01$ versus No yeast with elicitation by Dunnett's test.

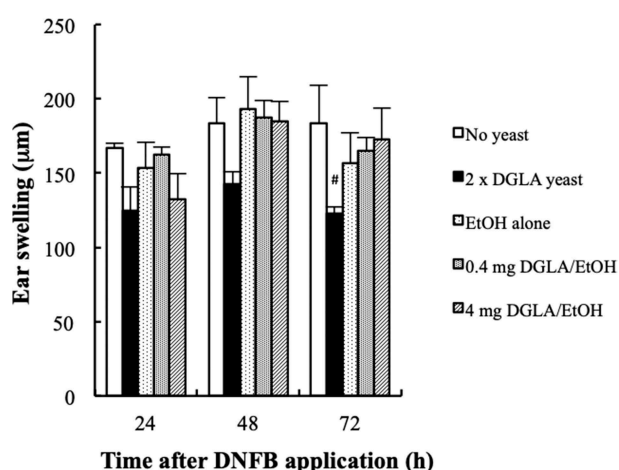


Figure 5. No suppression of inflammation by oral intake of purely chemical DGLA.

Mice were orally administered with 0, 0.4, and 4 mg/day of purified DGLA in 50 μ L of EtOH once a day. Mice with no yeast treatment (no yeast) and 2 x DGLA yeast-supplied mice (2 x DGLA yeast) were included as controls. After 7-day administration, DNFB was applied to ears of mice and ear thickness was measured. Ear swelling after DNFB application is shown as the means $\pm$ SE of four mice. # $p = 0.078$ versus no yeast control by one-way ANOVA with Dunnett's test.

acid. Since purified DGLA is very susceptible to oxidation, the contribution of cells to protect unsaturated fatty acid from degradation factor-like oxidation is quite likely. In addition to their oxidation during storage, we cannot neglect *in vivo* oxidation of PUFA in organs such as liver. Entire yeast cells may contribute to protect DGLA from such degradation. Ikeda et al. [20] examined the effects of dietary sesamin and sesaminol on the lipid peroxidation in the plasma and tissues of rats fed DHA, and suggested that dietary sesame lignans decrease lipid peroxidation as a result of elevating the α -tocopherol concentration in rats fed DHA. Yeast cells do not contain sesamin or sesaminol, but they might contain something that works similarly. For example, β -1,3-glucan of yeast has an antioxidation effect and it activates immune system [21,22]. Furthermore, yeast cells may facilitate the digestive and absorptive function or increase the coefficient of digestibility.

The safety of *S. cerevisiae* is traditionally recognized and *S. cerevisiae* is widely used as a nutrient

supplement as it highly nutritious and rich in protein, soluble fiber, and some minerals[23]. In our experiments, we did not notice any effect of yeast cells on the bodyweight of mice, but under favor of the recent increase in the consumption of yogurt expecting the probiotic effects of oral administration of lactobacillus on atopic dermatitis [24–28], similar usage of this type of *S. cerevisiae* for the prevention of dermatitis may be feasible in the future.

In conclusion, the present study demonstrated that a series of inflammatory reactions of ACD elicited by topical application of DNFB was significantly suppressed by the oral intake of the DGLA-producing yeast for 10 days, but not suppressed by 10 times the amount of purified DGLA. These findings also suggest that oral administration of the DGLA-producing yeast cells is considered to be a simple and efficient method to prevent ACD responses as well as ICD.

Author contributions

NW and HU conceived and designed experiments. ST and MO performed the experiments. NW and HU wrote the manuscript.

Disclosure statement

No potential conflict of interest was reported by the authors.

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