Liquiritigenin, a licorice flavonoid, helps mice resist disseminated candidiasis due to *Candida albicans* by Th1 immune response, whereas liquiritin, its glycoside form, does not.

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**A B S T R A C T**

Licorice (the root of *Glycyrrhiza* plant) has been used as an oriental herbal medicine for thousands of years. The licorice flavonoid components are reported to possess immunomodulatory activities. In this present study, we investigated the immunomodulatory effects of liquiritigenin (LG) and liquiritin (LQ), licorice flavonoid components, against disseminated candidiasis due to *Candida albicans*, a dimorphic fungus, that causes severe disease via hematogenous dissemination and local diseases such as vaginitis and thrush. Results showed that direct interaction of LG or LQ with *C. albicans* yeast cells resulted in no growth-inhibition, in vitro. When tested in a murine model of disseminated candidiasis, mice given LG intraperitoneally before intravenous challenge with live *C. albicans* yeast cells had similar mean survival times (MST) as untreated mice groups. On the contrary, mice given LG in the same manner as LQ above had longer MST than the untreated mice groups (P<0.05). In one experiment, 3 out of 5 LG-treated mice survived during the entire period of the 55-day observation. Furthermore, the 3 survivors were cured—shown by a lack of CFU (colony forming unit) in the kidneys. This protection was nullified when mice were pretreated with anti-CD4+ antibody before LG-treatment and challenge with the yeast. However, the protection was transferable by the CD4+ T cells isolated from LG-treated mice not infected with the yeast. In addition, mice given CD4+ T cells that were pre-treated with LG, in vitro were also protected against disseminated candidiasis. ELISA analysis revealed that in LG-treated mice IFNγ and IL-2 were dominantly produced compared to IL-4 and IL-10. When LG-given mice were treated with anti-mouse IFNγ, the protection was again nullified. Combined together, these results indicate that LG protects mice against disseminated candidiasis by the CD4+ Th1 immune response.

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1. Introduction

*Candida albicans*, a dimorphic fungus, is one of the leading causes of disseminated disease. Medical treatment for the infections mostly involves chemotherapeutic agents. For instance, amphotericin B and the azoles are mainly used in clinical situations, but toxicity of the drugs and resistance of the fungus are a major problem [1,2]. This has led many researchers to look for alternative ways of controlling the *C. albicans* infections. Among the alternative ways are fungal vaccine development [3,4], activation of innate immunity [5,6], and immune modulation by regulating immune responses [7,8]. These immunological ways for the prevention and treatment may have fewer problems with side effects and resistance, caused by the antifungal drugs, which can be beneficial in managing the fungal infections.

Helper (Th) lymphocytes are separated into Th1 and Th2 populations according to their profile of secreted cytokines [9]. A crucial step, which leads to recovery, in the inflammatory response to fungal infection is a Th1 immune response from CD4+ T cells [10]. In other words, severity of the fungal infection is correlated with dominance by either Th1 or Th2 response, and Th1 type dominance can reduce the severity of the infection. In fact, in the *C. albicans* infections, it has been shown that Th cell plays an important role in regulating the immune response to the *C. albicans* infection by secreting cytokines that can modulate activity of the immune effectors [11,12]. For example, our previous data show that ginsenoside Rg1, isolated from Panax ginseng [13], and daucosterol, a β-
sitosterol glycoside [14], resulted in protection of mice against disseminated candidiasis even though the two compounds had no antifungal activity during direct in vitro interaction with C. albicans yeast cells. In all these works, a dominance of the Th1 type immune response appears to be vital for the removal of the yeast cells in the mice [13,14]. In addition, these triterpenoidal compounds isolated from medicinal plants have an immunoregulatory activity that converts Th2 immune response to Th1 one in a murine model of disseminated candidiasis caused by C. albicans. Thus, finding natural compounds that have such an immunoregulatory activity is important in the regulation of the C. albicans infection. In this aspect, we examined flavonoidal compounds that are ubiquitously found in the plant kingdom.

Licorice root has been widely used for many centuries as an herbal medicine, especially in Asia and as a flavoring component in food products [15]. Licorice root contains flavonoids and triterpenoids [16–18]. A major flavonoid isolated from licorice root is liquiritin (LQ), which is glycosidic form of liquiritigenin (LG) [20]. LG is a metabolite of LQ and is known to be actually absorbed into the body [20,21]. The chemical structures of LQ and LG are shown in the Fig. 1. These flavonoids possess various biological effects such as antiallergic, antitussive [22,23] and immunomodulating [24] activities. However, the immunomodulating activity of LQ and LG on CD4+ T cells has not been clear until now.

In this study, we determined if the flavonoid, LQ, and its active metabolite form, LG, have the immunoregulatory activity like ginsenoside Rg1 and daucosterol that leads to the recovery from the C. albicans infection. Here, we show that Th1 conversion by LG, but not LQ, conferred resistance to mice by inducing the dominant Th1 type differentiation.

2. Materials and methods

2.1. Organisms and culture conditions

All strains of C. albicans, CA-1, A9, and 3153A, previously characterized [13,14,25–27], were grown in glucose-yeast extract-peptone (GYEP) broth at 37 °C as mentioned before [13,14]. For infection of mice, blastosconidial form of C. albicans grown in GYEP broth was collected, washed with cold sterile Dulbecco's phosphate-buffered saline (DPBS; Sigma, St. Louis, USA) solution, and enumerated with use of hemocytometer to obtain desired numbers of yeast cells.

2.2. Mice

BALB/c female mice at 6 weeks of age (Charles River Lab. USA) were used. Mice were maintained in the animal facility under the Dongduk Women's University's regulation.

Fig. 1. Structures of liquiritigenin (LG) and liquiritin (LQ). LQ:1H-NMR (250 MHz, DMSO-d6): δ 2.66 (1H, d, J = 2.7, 16.7 Hz, H-3), 3.07–3.40 (5H, m, H-2′, 3′, 4′, 5′, 3), 3.46 (1H, dd, J = 5.4, 11.2 Hz, H-6′) and 3.68 (1H, dd, J = 3.8, 11.5 Hz, H-6″). 4.88 (1H, d, J = 7.1 Hz, H-1″), 5.32 (1H, dd, J = 2.5, 12.6 Hz, H-2″, H-1), 3.44 (1H, d, J = 2.1 Hz, H-8″), 6.50 (1H, dd, J = 2.1, 8.7 Hz, H-6), 7.06 (2H, d, J = 8.7 Hz, H-5′, 5″), 7.44 (2H, d, J = 8.7 Hz, H-2′, H-6′), 7.64 (1H, d, J = 8.7 Hz, H-1), 7.06 (2H, d, J = 8.7 Hz, H-5′, 5″), 7.44 (2H, d, J = 8.7 Hz, H-2′, H-6′), 7.64 (1H, d, J = 8.7 Hz, H-1). 13C-NMR (62.5 MHz, CD3OD): δ 178.9 (C-2), 170.0 (C-2′), 132.6 (C-5), 110.8 (C-6), 164.9 (C-7), 102.8 (C-8), 131.3 (C-9), 113.8 (C-10), 132.6 (C-1′), 128.2 (C-2′), 116.4 (C-3′), 157.7 (C-4′), 116.4 (C-5′), 128.2 (C-2′), 100.5 (C-1″), 73.4 (C-2″), 76.8 (C-3″) 69.9 (C-4″), 77.3 (C-5″), 60.9 (C-6″). LG:1H-NMR (250 MHz, CD3OD): δ 2.85 (1H, dd, J = 13.0, 16.9 Hz, H-3), 2.66 (1H, dd, J = 2.9, 16.9 Hz, H-3), 5.34 (1H, dd, J = 2.9, 13.0 Hz, H-2), 6.34 (1H, dd, J = 2.3 Hz, H-8), 6.48 (1H, dd, J = 2.3, 8.7 Hz, H-6), 6.81, 7.30 (each 2H, d, J = 8.6 Hz, H-2′, 3′, 5″, 6′), 7.71 (1H, d, J = 8.7 Hz, H-5′), 13C-NMR (62.5 MHz, CD3OD): δ 44.9 (C-3), 81.0 (C-2′), 103.8 (C-8), 111.7 (C-6), 114.9 (C-10), 116.3 (C-3′, 5′), 129.0 (C-2′, 6′), 129.9 (C-5), 131.3 (C-1′), 158.9 (C-4′), 165.3 (C-5), 166.7 (C-7), 193.6 (C=O).

2.3. Liquiritin (LQ) and liquiritigenin (LG)

Isolation of LQ and LG was done by referencing the previous works of others [28,29]. In brief, the dried roots of Glycyrrhiza uralensis (a family of Leguminosae; 10 kg) were powdered and extracted with 100% methanol (50 l) at room temperature. The roots were identified by Prof. Je-Hyun Lee (College of Oriental Medicine, Dongguk University, Gyeongju, South Korea). A voucher specimen has been deposited at the College of Pharmacy, Yeungnam University, South Korea. After the methanol extract was concentrated by evaporation, 2.6 kg of residue was suspended in 3.51 of distilled water (3.5 l) and partitioned with the same volume of methylene chloride (CH2Cl2). The partitioning procedure was repeated two more times. The CH2Cl2 soluble part (230 g) was chromatographed on a silica gel (Sigma) column eluting with a gradient of mixture of n-hexane and ethyl acetate at ratios of 100:0, 98:2, 95:5, 90:10, 85:15, and 80:20, respectively. Each volume of the gradients was 5 l. By this elution, sixteen fractions were obtained. From those sixteen fractions, fraction 9 (2400 ml, n-hexane-ethyl acetate, 90:10) was rechromatographed over the silica gel column with mixture of CH2Cl2 and methanol to yield liquiritigenin, purity of which was 100% [28]. Additionally, fraction 14 (3600 ml, n-hexane-ethyl acetate, 80:20) was rechromatographed over silica gel column using an isocratic solvent of mixture of CH2Cl2 and methanol (21) to obtain liquiritin (800 mg; purity 99%) [29]. These two fractions were each recrystallized—producing an amorphous white powder. The fractions’ spectroscopic and physical data were directly compared to those of authentic samples as reported by others [28,29].

The isolated compound was tested for the presence of the endotoxin by following the manufacturer’s guidelines for the Limulus amebocyte lysate test (E-Toxate Kit; Sigma). Results showed that the LG and LQ had no endotoxin content under the condition of the commercial kit (Escherichia coli O55:85 lipopolysaccharide was the positive control). In addition, prior to use in experiments, the LG and LG that were dissolved in sterile DPBS containing 0.05% DMSO at a desired concentration and filter-sterilized (a pore size = 0.2 μM; Sartorius, Goettingen, Germany) were each inoculated on a blood agar plate (Korean Culture Media, Seoul, Korea) to check for any microbial contamination. It was found that no microbial contamination was observed in the LG and LQ.

2.4. Anti-candidal activity, in vitro

To determine antifungal activity of LQ and LG on C. albicans growth, broth susceptibility method was used as previously described [26,30]. In brief, 100 μl of C. albicans suspension (5 × 10⁶ yeast cells/ml) was put into a well of a 96 well plate ( Falcon). To designated wells, 100 μl of LG or LQ (each at 100 μM as a final concentration) prepared in...
sterile DPBS containing 0.05% DMSO was added. A negative control well received a same volume of diluent (DPBS) only. As a positive control, fluconazole diluted in DPBS containing 0.05% DMSO was put into a well. All plates were incubated at 37 °C for 48 h. After the incubation, 100 μl from the well was inoculated on Mycobiotic agar (Difco, Sparks, MA) and incubated at the same culture condition. Twenty-four hours later, colony forming unit (CFU) was enumerated.

In this experiment, all the three strains of C. albicans were tested.

2.5. Effects of LQ and LG on mice against disseminated candidiasis

Effect of each of the compounds was determined in a murine model of disseminated candidiasis as previously characterized and described [3,13,14]. In experiments, mice were given 200 μl of LQ or LG (100 μg/ml) dissolved in DPBS plus 0.05% DMSO, i.p., twice during a three-day interval. Twenty-four hours after the last booster, these animals were challenged, intravenously (i.v.), with viable C. albicans (strain CA-1) yeast cells (5 × 10^6 cells per mouse). Control mice received diluent instead of LQ or LG before the challenge. Their survival differences were calculated for statistical significance. In addition, numbers of CFUs per gram of kidney tissue in mice, which were identically set up as the mice groups above, were counted. In disseminated candidiasis, the kidney is a target organ; therefore the number of C. albicans CFU in kidney tissue can be used as an indicator of disease severity [31,32]. The CFU determinations were done 48 h after the challenge by homogenizing the kidneys with glass tissue homogenizer as described previously [3,14].

From our previous work [14], the amount of the 0.05% DMSO on mice against the disseminated candidiasis had no influence on C. albicans growth.

2.6. Isolation of LG-treated or LQ-treated CD4+ T cells (LGCD4T or LQCD4T) in mice

Mice (BALB/c strain) were treated with LG or LQ (40 μg per mouse in a 200 μl volume), respectively, by intraperitoneal (i.p.) route twice in a three-day interval. Three days after the last booster, spleens from the LG- or LQ-treated mice were each harvested and were put into ice-cold Hank’s salt solution. Isolation of these CD4+ T cells was done with mouse T cell CD4 subset column kit (Cat# MCD4C-100, R&D Systems) and then single cell suspensions were prepared based on our previous work [13] that was modified from other's procedure [33]. In brief, the suspensions were passed through a sterile steel screen and were pelleted by centrifugation. Red blood cells were removed by lysing them for 5 min with ACK lysing buffer containing 0.15 M NH4Cl, 1 mM KHCO3, and 0.1 mM Na2EDTA. The collected cells were then passed through the CD4+ T cell subset column (R & D Systems, Minneapolis, MN, USA). After fractionation on the column, viability of the CD4+ T cell was measured with trypan blue solution (Sigma). By the single cell suspension procedure, the adherent cells to the culture plate were discarded. For use as a negative control, CD4+ T cell from normal mice (naive CD4T) that received only diluent by the same route was collected by the identical procedure as the above. Each preparation of these T cells was resuspended at a concentration of 10^7/ml in sterile DPBS.

Purity of the isolated CD4+ T cell was measured by FACS (fluorescence-activated cell sorter) analysis using fluorescein-conjugated anti-CD4 (PharMingen) specific for mouse CD4. According to the manufacturer’s information, the purity of recovered rates of CD4+ T cells was ranged from 84% to 91%.

2.7. Detection of cytokine production from the LGCD4T

For determination of the cytokine secretions of IFN-γ, IL-2, IL-4, and IL-10 from the isolated CD4+ T cells in the Section 2.6, each of the CD4+ T cell preparations was plated in 24-well dishes at a concentration of 5 × 10^5 cells/well and stimulated by mouse anti-CD3 MAb (PharMingen, San Diego, CA) for 48 h in 5% CO2 at 37 °C as previously described [13]. After the stimulation, the supernatants from the CD4+ T cells were separately collected and stored at −20 °C until tested by ELISA kits (R & D Systems).

2.8. Effect of LGCD4T on CD4+ T cell-depleted mice (CD4DM) against disseminated candidiasis

To confirm the protective effect of LG against the disseminated candidiasis, mice were pretreated with Gk1.5 rat anti-mouse CD4 antibody (150 μg/mouse/dose) twice in a three-day interval for depletion of CD4+ T cell in the animals. Three days after the last treatment, these mice were divided into two groups. One group received LGCD4T (0.3 ml/mouse at 10^7 T cells) and the other group was given naïve CD4T via i.v.-administration. Twenty-four hours after the T cell inoculation, the two groups of the mice and a normal mice group (control) were all infected with viable yeast cells of C. albicans CA-1 as described above, and their survival rates were measured. The anti-CD4 antibody was isolated from the GK 1.5 cell line (ATCC, Rockville, MD) as previously described [13].

Prior to the experiment, the in vivo CD4+ T cell depletion after the anti-CD4 antibody treatment was confirmed by FACS analysis of spleen cells using fluorescein-conjugated antiserum (PharMingen) specific for mouse CD4.

2.9. Effect of anti-IFNγ on LG-treated mice against the disseminated disease

Mice pretreated with LG as described in Section 2.5 were given anti-mouse IFNγ antibody (R46A2; ATCC) via i.p. injection at 0.5 mg/mouse in a volume of 0.2 ml 24 h before infection with C. albicans (strain CA-1) [34]. For a positive control, a group of mice received the same amount of LG, but no anti-IFNγ. The survival times of these mice were measured.

2.10. Effect of CD4+ T-cells treated with LG, in vitro, against disseminated candidiasis

To determine if LG has a direct effect on the protection against the disseminated disease, isolated CD4+ T cells (5 × 10^7 cells/ml), by the procedure as described in Section 2.6, were put in culture medium (RPMI (Sigma) with 10% FBS (Hyclone) plus penicillin-streptomycin prep for the cell culture (Invitrogen-Gibco)] and mixed with LG (a 100 μM final concentration). After incubation, the LG-treated CD4+ T cells were washed with sterile DPBS three times by centrifugation in order to remove LG residue in the preparation. The washed CD4+ T cells that were suspended at a concentration of 10^7/ml in sterile DPBS were administered into mice via i.v.-route. The dose per mouse of CD4+ T cells was 150 μl or 300 μl, respectively, at 10^7 T cells/ml. Control mice groups received either naïve CD4T (300 μl/mouse at 10^7 T cells/ml) that were untreated with the LG or diluent (DPBS) by the identical route. Twenty-four hours

### Table 1

<table>
<thead>
<tr>
<th>Treatment Name of C. albicans strain</th>
<th>CA-1</th>
<th>A-9</th>
<th>3153A</th>
</tr>
</thead>
<tbody>
<tr>
<td>LG</td>
<td>(27.8 ± 3.57) × 10^3</td>
<td>(29.9 ± 4.02) × 10^3</td>
<td>(31.9 ± 5.02) × 10^3</td>
</tr>
<tr>
<td>LQ</td>
<td>(31.2 ± 1.17) × 10^3</td>
<td>(28.0 ± 1.98) × 10^3</td>
<td>(30.0 ± 2.16) × 10^3</td>
</tr>
<tr>
<td>Diluent only</td>
<td>(26.3 ± 1.52) × 10^3</td>
<td>(275.3 ± 2.32) × 10^3</td>
<td>(28.1 ± 3.28) × 10^3</td>
</tr>
<tr>
<td>Fluconazole</td>
<td>(6.70 ± 0.21) × 10^2</td>
<td>(3.00 ± 1.57) × 10^3</td>
<td>(2.18 ± 0.02) × 10^2</td>
</tr>
</tbody>
</table>

[Unit: CFU/ml].

Note:

The (*) symbol indicates mean ± S.E.

The values presented were determined at 100 μM as a final concentration.
after the T cell inoculation, these mice groups rol) were all infected with viable yeast cells of \textit{C. albicans} CA-1 as described above, and their survival rates were measured.

### 2.11. Statistics

Statistical significance of differences in survival times was calculated by the Kaplan–Meier method (New Statistic for Windows; SPSS, Chicago, USA). The paired \( t \) test was used to make comparison between vehicle- and LQ or LG-treated mice groups when applicable. Differences between the two groups were considered statistically significant if \( P \) value was less than 0.05.

### 3. Results

#### 3.1. Structure elucidation

The isolated compounds from \textit{G. uralensis} were determined as LQ and LG, respectively (Fig. 1).

#### 3.2. LQ and LG have no antifungal effect on \textit{C. albicans} by direct contact

To determine if LQ and LG inhibit growth of \textit{C. albicans} yeast cells, broth susceptibility test was done. Results showed that both LQ and LG had no growth-inhibition on all the test \textit{C. albicans} strains, respectively, whereas fluconazole that was used for a positive control inhibited \textit{C. albicans} growth (Table 1).

#### 3.3. The LG treatment, but not LQ, protects mice against disseminated candidiasis

To determine if LQ or LG is protective to the disseminated disease, the survival rates of the mice given LQ or LG before i.v.-challenge with \textit{C. albicans} yeast cells were assessed. The resulting survival curves were plotted. The LG-treated mice group had MST values of 40.8 ± 20.9 days, whereas the untreated (control) mice had MST values of 14.2 ± 3.9 days. The LG-treated mice group survived approximately 26 days longer which is a significant difference from control mice group (\( P < 0.01 \)). In contrast, the MST value from mice given LQ was as almost the same as MST value from the control, indicating that LQ had no protective activity. The measurement was terminated at day 55. This experiment was repeated three times, and the pattern of the survival rates was similar. MST stands for mean survival times.

![Fig. 2. LG treatment enhances resistance of mice against disseminated candidiasis. Mice given LG by i.p.-route were challenged, i.v. with viable \textit{C. albicans} yeast cells. The resulting survival curves were plotted. The LG-treated mice group had MST values of 40.8 ± 20.9 days, whereas the untreated (control) mice had MST values of 14.2 ± 3.9 days. The LG-treated mice group survived approximately 26 days longer which is a significant difference from control mice group (\( P < 0.01 \)).](image)

![Fig. 3. LG, but not LQ, protects mice against the disseminated disease. By the identical way as described for the Fig. 1 legend, mice were treated with LG before i.v.-challenge with \textit{C. albicans}. Forty-eight hours after the challenge, the resulting kidney candidal CFU per gram of tissue was determined. The LG-treated mice resulted in less number of kidney CFU than untreated control mice groups. Differences between the CFU values from LG-treated mice and CFU values from the control were significant (\( P < 0.01 \)). However, the CFU values from LQ-given mice resulted in almost the same values as the control mice groups. Bars, standard errors. Each group contained five mice.](image)

![Fig. 4. Predominant Th1 cytokines is induced in the LGCD4T culture (A), whereas such dominance is absent in the LQCD4T (B). Splenic CD4 T cells were isolated from mice that were treated with the LG twice every three days (LGCD4T). Culture supernatants of the LGCD4T and CD4+ T cell from normal mice (naïve CD4T) were assayed for IFN\( \gamma \), IL-2, IL-4 and IL-10, respectively, by ELISA. The ELISA analysis revealed that Th1 type cytokines (IFN\( \gamma \) and IL-2) were predominantly produced in the LGCD4T as compared with Th2 type cytokines of IL-4 and IL-10 productions. The naïve CD4T produced no such cytokines, displaying only background of absorbance. The similar observation like the naïve CD4T (control) values was made with LQCD4T. Values are mean ± S.E. for three replicates obtained with pooled cells from five mice per group.](image)
The CD4+ T cell-depleted mice that received LGCD4T by adoptive transfer recovered their resistance to disseminated candidiasis. The CD4+ T cell-depleted mice (CD4DM) were grouped into two groups. One group was treated with LGCD4T (CD4DM/LGCD4T), and the other group received naïve CD4T (CD4DM/Naïve CD4T). Normal mice that received only diluent (NM/Diluent) were used as a negative control. All of these animals were challenged i.v. with 5×10⁵ viable yeast cells, and their resistance to the disseminate disease was assessed. The assessment showed the subgroup of CD4DM/LGCD4T survived longer than CD4DM/Naïve CD4T. The survival rates from NM/Diluent control mice groups (Fig. 2). That is, all of the five untreated mice died by day 19, whereas LG-treated mice groups had mean survival times (MST) of 40.8±20.9 (MST+S.E.) days. Three of the LG-treated mice survived during the entire duration of 55 day-observation (Fig. 2). The MST value (11.6±3.6 days) from LG-treated mice was even less than MST value of the control group, resulting in approximately 3 days difference. The experiment was conducted, and all experiments led to similar results. Thus, we emphasized testing only LG for the following evaluations.

### 3.4. Evaluation of Th1 and Th2-type cytokine

The in vitro expression of cytokines involved in type 1 (IFNγ and IL-2) and type 2 (IL-4 and IL-10) immune responses by LG- or LQ-stimulated T-cells was each evaluated. Results showed that the stimulation evoked productions of IFNγ and IL-2 more than those of IL-4 and IL-10 from the LGCD4T, revealing predominant induction of the Th1-type immune response. The difference between the productions of the type 1 and the type 2 cytokines was significant (P<0.01). In case of naïve CD4T without LG stimulation (a negative control group), there were only backgrounds of optical density in the ELISA assay, whose ELISA values were very similar as the values assayed with LG-treated CD4+ T cells (LQCD4T) (Fig. 4).

### 3.5. Administration of the LGCD4T recovers impaired resistance of the CD4DM to levels observed in LG-treated mice

To determine if splenic CD4+ T cell stimulation by LG is responsible for the protection, the CD4+ T cell-depleted mice were reconstituted with LGCD4T by adoptive transfer before challenge with C. albicans. Control CD4+ cell-depleted mouse groups received naïve CD4T, instead of LGCD4T. The survival rates of the two mice groups were then compared with survivability of CD4+ T cell competent normal mice after the challenge with C. albicans. Results showed that CD4+ T cell-depleted mice that received naïve CD4T had a MST value of 8.6±3.2 which is similar to MST value from the anti-CD4-untreated normal animals that were given diluent (NM/Diluent) (9.9±4.5 days) (Fig. 5). However, CD4+ T cell-depleted mice reconstituted with LGCD4T had increased MST value of 30.3±9.8 days (Fig. 5). This difference was statistically significant (P<0.05). These survival
patterns were similar to the survival patterns of normal mice with or without LG treatment as shown in Fig. 2.

The FACS analysis showed that the anti-CD4 treatment of mice almost entirely depleted the CD4+ T cells (data not shown).

3.6. The LG induced protection of mice by its direct activation of CD4+ T cells

To determine if protective effect of the LG is mediated by direct activation of CD4+T cells, survival rates of mice that were given LG-stimulated CD4+ T cells (LG/CD4T), in vitro, by adoptive transfer before the C. albicans infection were examined. Results showed that all mice groups that received the LG/CD4T survived longer than diluents-given (control) mice groups (Fig. 6). This difference was statistically significant (P<0.05). Although all the animals treated with the LG/CD4T at 150 μl or 300 μl, respectively died within 19 days and 30 days, the protective effect was dose-dependent (Fig. 6). The survival rates from naïve CD4+ T cells (naïve CD4T)-given mice groups had almost the same survival rates, corresponding to MST values of 8.4±2.6 days and 9.2±3.2 days, respectively (Fig. 6), displaying that the naïve CD4 + T cells alone had no such protective activity.

Repeated experiments resulted in the similar data.

3.7. Treatment of anti-IFNγ antibody abolishes the protection of LG-treated mice

Effect of anti-IFNγ antibody on LG-treated mice against the disseminated disease was determined. Results showed that the survivability of the animals was abolished when compared to survival rates of mice treated with only LG (Fig. 7). This abolishment resulted in an almost complete drop of survivability to the level of survival rates measured from LG-untreated mice groups as shown in Fig. 2.

4. Discussion

A proper balance between Th1 and Th2 immune responses is considered to play a key role in the controlling of some infections [9–12]. In C. albicans-infections, Th1 response seems to be beneficial for the clearance of the mycotic infection. The Th1 response that induces IFNγ and IL-2 contributes cell-mediated immunity, which enhances killing capacity of the various effector cells such as cytotoxic T-cells, NK cells, and activated macrophage against these effectors invaded by C. albicans [35–37]. However, such a situation cannot be easily developed in the host with a predominance of Th2 immune response producing IL-4 and IL-10, which suppress Th1 type response. For instance, neutralization of IL-4 by anti-IL-4 antibody displays protection against disseminated candidiasis [34,36]. This indicates that discovering components that can stimulate Th1 response and/or antagonize Th2 response can be a remarkable way for controlling the disseminated disease.

In this study, we demonstrated the immunomodulatory effects of LQ and LG (aglycone of LQ) against disseminated candidiasis. Both of the compounds had no killing activity when they were directly in contact with C. albicans yeast cells as evidenced from the in vitro susceptibility test. Only LG, however, in the mouse body was C. albicans contact with the compounds had no killing activity when they were directly in the presence of LQ and LG (aglycone of LQ) against disseminated candidiasis. Both of these compounds were used to test if the LG could directly activate CD4+ T cell activation, which consequently induced the protective Th1 immunity. For the determination of this point, preparation of CD4 + T cells that were pre-treated with the LG, in vitro, was applied. Results from the experiment revealed that the preparation indeed enhanced resistance of mice against the disseminated disease. However, this protection appears to be partial when compared to the pattern of protection resulted from the LG was administered into mice and infected with the yeast cells. This observation made us draw a speculation. That is, administration of insufficient numbers of the LG-activated CD4 + T cells into the test animals because this circumstance might cause poor production of IFNγ, a major cytokine production from Th1 type CD4 + T cells, which conceivably fails blocking the induction of Th2 type cytokine such as IL-4 and IL-10 that aggravate C. albicans infection. As mentioned above, macrophage activity might be limited under the circumstance of insufficient amount of IFNγ, thus diminishing its APC function and antifungal activity. For the latter case, we even demonstrated that the protection could be abolished by removal of IFNγ in the LG-treated mice, but administration of anti-IFNγ into LG-treated mice restored the protection. Investigation might be needed to determine how T cells influence macrophage via the cytokine in the process of the protection. However, doing the investigation in the present study is beyond our scope. Combined together, we concluded that the LG can induce protection against the disseminated candidiasis by direct activation of CD4 + T cells resulting in the Th1 immune response.

Over all, LG, a licorice flavonoid, has an immunomodulating activity that mediates induction of dominant Th1 type cytokine production from the activated CD4 + T cell, which appears to be responsible for the protection of mice against disseminated candidiasis. From these data, it can be predictable that flavonoids from other plants besides the licorice would have similar activity like the LG. However, permeability of any test flavonoids should also be determined under in-vivo conditions.
Currently, we are investigating if LG has adjuvant activity that can promote production of IgG2a anti-Candida antibody by B-lymphocytes.

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References


