



## 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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### ABSTRACT

Licorice (the root of *Glycyrrhizae* 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 LQ 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 nulled 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 IFN $\gamma$  and IL-2 were dominantly produced compared to IL-4 and IL-10. When LG-given mice were treated with anti-mouse IFN $\gamma$ , the protection was again nulled. 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 immunolo-

gical 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.

T 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  $\beta$ -

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sitosterol glycoside [14], resulted in protection of mice against disseminated candidiasis even though the two compounds had no anticandidal 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]. In fact, the flavonoids and triterpenoid are the two major bioactive components [19]. 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, blastoconidial 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.

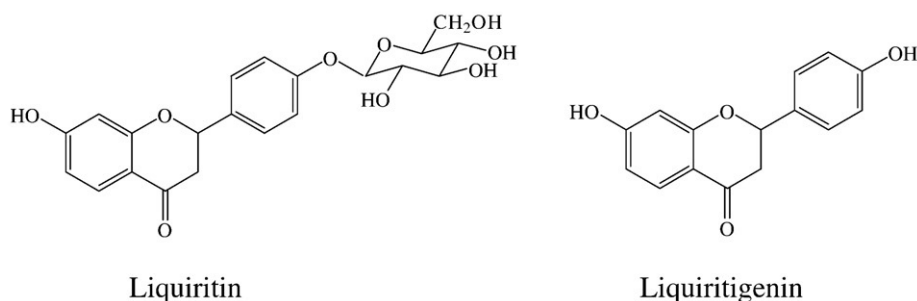
### 2.3. Liquiritin (LQ) and liquiritigenin (LG)

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 3.5 l of distilled water (3.5 l) and partitioned with the same volume of methylene chloride (CH<sub>2</sub>Cl<sub>2</sub>). The partitioning procedure was repeated two more times. The CH<sub>2</sub>Cl<sub>2</sub> 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 CH<sub>2</sub>Cl<sub>2</sub> 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 CH<sub>2</sub>Cl<sub>2</sub> and methanol (2 l) 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 amoebocyte lysate test (E-Toxate Kit; Sigma). Results showed that the LQ and LG had no endotoxin content under the condition of the commercial kit (*Escherichia coli* O55:B5 lipopolysaccharide was the positive control). In addition, prior to use in experiments, the LQ 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 anticandidal 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 × 10<sup>6</sup> 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



**Fig. 1.** Structures of liquiritigenin (LG) and liquiritin (LQ). LQ: <sup>1</sup>H-NMR (250 MHz, DMSO-*d*<sub>6</sub>): δ 2.66 (1H, dd, *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"), 5.52 (1H, dd, *J* = 2.5, 12.6 Hz, H-2), 6.34 (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-3', 5'), 7.44 (2H, d, *J* = 8.7 Hz, H-2', 6'), 7.64 (1H, d, *J* = 8.7 Hz, H-5); <sup>13</sup>C-NMR (62.5 MHz, DMSO-*d*<sub>6</sub>): δ 78.9 (C-2), 43.4 (C-3), 190.2 (C-4), 132.6 (C-5), 110.8 (C-6), 164.9 (C-7), 102.8 (C-8), 163.3 (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-6'), 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: <sup>1</sup>H-NMR (250 MHz, CD<sub>3</sub>OD): 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, d, *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); <sup>13</sup>C-NMR (62.5 MHz, CD<sub>3</sub>OD): 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'), 165.5 (C-9), 166.7 (C-7), 193.6 (C=O).

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% DPBS 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 ( $5 \times 10^5$  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 Hanks'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 NH<sub>4</sub>Cl, 1 mM KHCO<sub>3</sub>, and 0.1 mM Na<sub>2</sub>EDTA. 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 adhesive cells to the culture plate were discarded. For use as a negative control, CD4+ T cell from normal mice (naïve 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 antisera (PharMingen) specific for mouse CD4. According to the manufacture'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  $5 \times 10^6$  cells/well and stimulated by mouse anti-CD3 MAb (PharMingen,

San Diego, CA) for 48 h in 5% CO<sub>2</sub> 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 antisera (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 ( $5 \times 10^7$  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 final concentration of 1 µg/ml) for 2 h at 37 °C-5% CO<sub>2</sub> incubator. After the 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 resuspended 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**

Antifungal effect of the liquiritigenin (LG) and liquiritin (LQ) on *C. albicans* growth, *in vitro*.

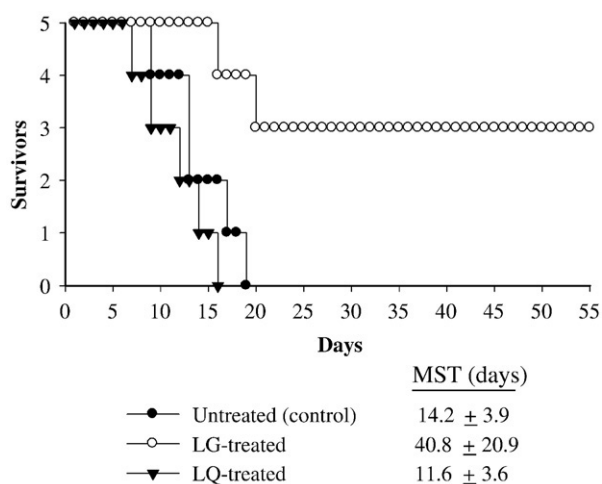
Treatment	Name of <i>C. albicans</i> strain		
	CA-1	A-9	3153A
LG	(27.8 ± 3.57)* × 10 <sup>3</sup>	(29.0 ± 4.02) × 10 <sup>3</sup>	(31.0 ± 5.02) × 10 <sup>3</sup>
LQ	(31.2 ± 1.17) × 10 <sup>3</sup>	(28.0 ± 1.98) × 10 <sup>3</sup>	(30.0 ± 2.16) × 10 <sup>3</sup>
Diluent only	(26.3 ± 1.52) × 10 <sup>3</sup>	(27.5 ± 2.32) × 10 <sup>3</sup>	(28.1 ± 3.28) × 10 <sup>3</sup>
Fluconazole	(6.70 ± 0.21) × 10 <sup>2</sup>	(3.00 ± 1.57) × 10 <sup>2</sup>	(2.18 ± 0.02) × 10 <sup>2</sup>

[Unit: CFU/ml].

Note:

The (\*) symbol indicates mean ± S.E.

The values presented were determined at 100 µM as a final concentration.

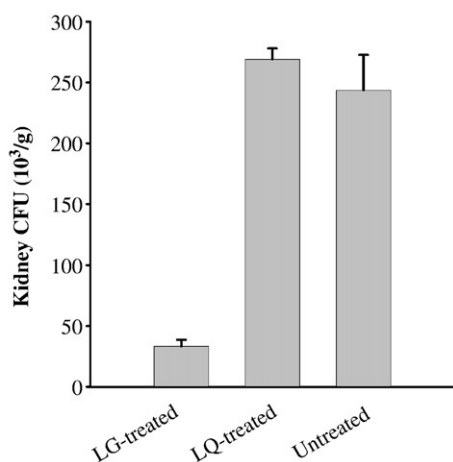


**Fig. 2.** LG treatment enhances resistance of mice against disseminated candidiasis. Mice given LG by i.p.-route were challenged, i.v. with viable *C. albicans* yeast cells. The resulting survival curves were plotted. The LG-treated mice group had MST values of  $40.8 \pm 20.9$  days, whereas the untreated (control) mice had MST values of  $14.2 \pm 3.9$  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.

after the T cell inoculation, these mice groups (rol) were all infected with viable yeast cells of *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.



**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 *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 the 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.

## 3. Results

### 3.1. Structure elucidation

The isolated compounds from *G. uralensis* were determined as LQ and LG, respectively (Fig. 1).

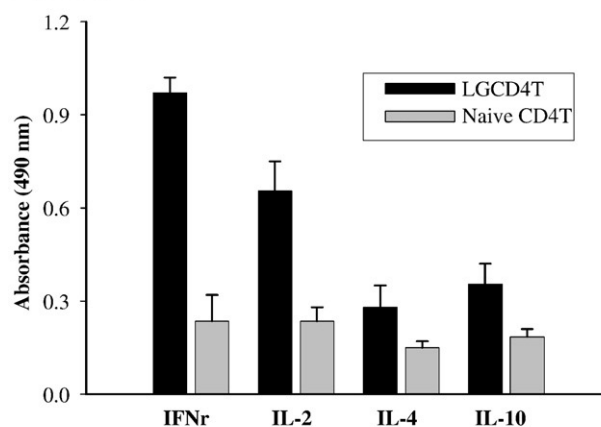
### 3.2. LQ and LG have no antifungal effect on *C. albicans* by direct contact

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

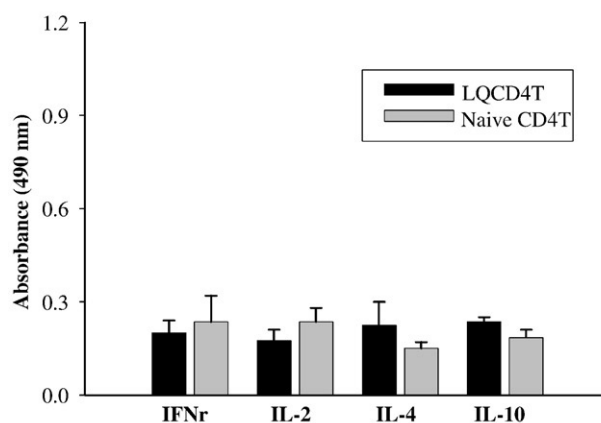
### 3.3. The LG treatment, but not LQ, protects mice against disseminated candidiasis

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 *C. albicans* yeast cells were assessed. The resulting survival curves

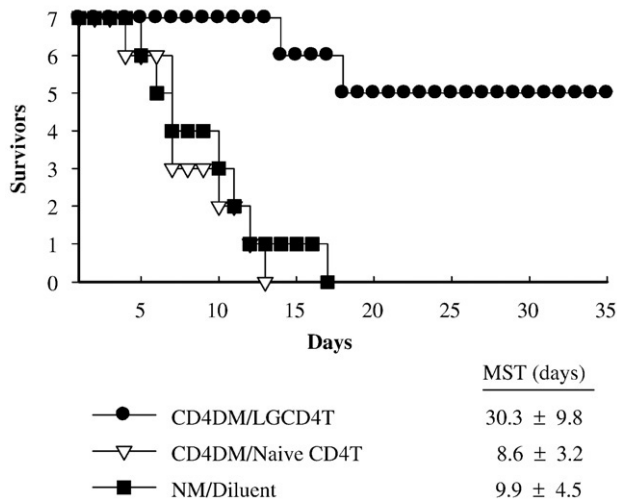
### A) LGCD4T



### B) LQCD4T

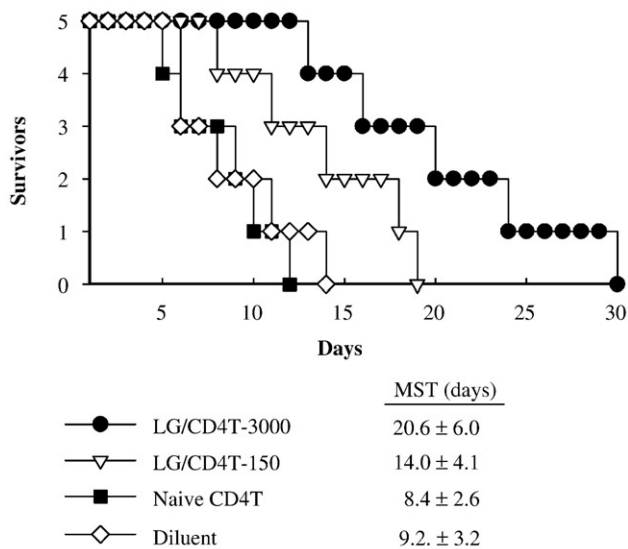


**Fig. 4.** Predominant Th1 cytokines is induced in the LGCD4T culture (A), whereas such dominance is absent in the LQCD4T (B). Splenic CD4 T cells were isolated from mice that were treated with the LG twice every three days (LGCD4T). Culture supernatants of the LGCD4T and CD4+ T cell from normal mice (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  $\pm$  S.E. for three replicates obtained with pooled cells from five mice per group.



**Fig. 5.** The CD4<sup>+</sup> T cell-depleted mice that received LGCD4T by adoptive transfer recovers their resistance to disseminated candidiasis. The CD4<sup>+</sup> 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/Naive CD4T). Normal mice that received only diluent (NM/Diluent) were used as a negative control. All of these animals were challenged i.v. with  $5 \times 10^5$  viable yeast cells, and their resistance to the disseminate disease was assessed. The assessment showed the subgroup of CD4DM/LGCD4T survived longer than CD4DM/Naive CD4T. The survival rates from NM/Diluent group were almost same as survival rates from CD4DM/Naive CD4T. Difference between CD4DM/LGCD4T and CD4DM/Naive CD4T was significantly different ( $P < 0.05$ ). Each group contained seven mice. MST stands for mean survival times.

displayed that the LG-treated mice survived longer than DPBS-given 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  $40.8 \pm 20.9$  (MST + S.E.) days. Three of the LG-treated mice survived during the entire duration of 55 day-observation (Fig. 2). The differences between the two groups were statistically significant ( $P < 0.01$ ). The candidal CFU evaluation showed that the LG-treated mice had approximately 84% reduction of CFU in kidney tissue as



**Fig. 6.** The CD4<sup>+</sup> T cells treated with LG *in vitro* (LG/CD4T) help mice resist against disseminated candidiasis. The mice groups given LG/CD4T survived longer than mice groups that received either diluents or LG-naïve CD4<sup>+</sup> T cells (naïve CD4T) during an entire period of 30-day observation. In addition, this protection was in a dose-dependent fashion. Difference between LG/CD4T-received mice and naïve CD4<sup>+</sup>T-given animals was statistically significant ( $P < 0.05$ ). Repeated experiments resulted in similar data. MST stands for mean survival times. Unit for the numbers are in microliter.

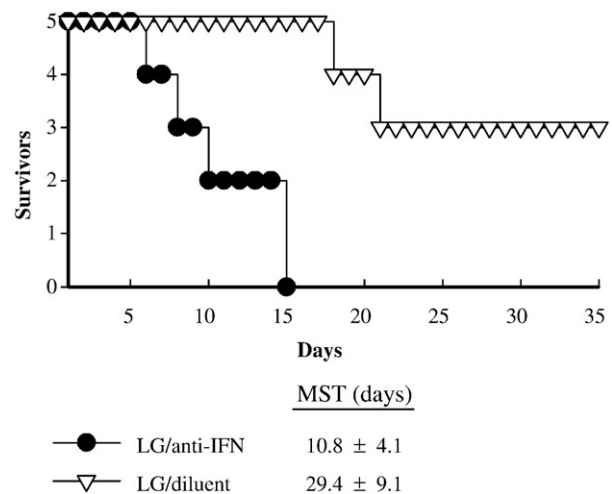
compared to CFU values from diluent (DPBS plus 0.05% DMSO)-given control mice, whereas CFU values from LQ-treated animals were slightly greater than the CFU values from the control mice groups (Fig. 3). These CFU-value differences between LG-treated groups and the controls were also statistically significant ( $P < 0.01$ ). In contrast to LG, LQ had no protective activity (Fig. 2). The MST value ( $11.6 \pm 3.6$  days) from LQ-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 (IFN $\gamma$  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 IFN $\gamma$  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 LQ-treated CD4<sup>+</sup> 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<sup>+</sup> T cell stimulation by LG is responsible for the protection, the CD4<sup>+</sup> T cell-depleted mice were reconstituted with LGCD4T by adoptive transfer before challenge with *C. albicans*. Control CD4<sup>+</sup> cell-depleted mice group received naïve CD4T, instead of LGCD4T. The survival rates of the two mice groups were then compared with survivability of CD4<sup>+</sup> T cell competent normal mice after the challenge with *C. albicans*. Results showed that CD4<sup>+</sup> T cell-depleted mice that received naïve CD4T had a MST value of  $8.6 \pm 3.2$  days which is similar to MST value from the anti-CD4-untreated normal animals that were given diluent (NM/Diluent) ( $9.9 \pm 4.5$  days) (Fig. 5). However, CD4<sup>+</sup> T cell-depleted mice reconstituted with LGCD4T had increased MST value of  $30.3 \pm 9.8$  days (Fig. 5). This difference was statistically significant ( $P < 0.05$ ). These survival



**Fig. 7.** The protection is abolished when LG-pretreated mice were given anti-mouse IFN $\gamma$  prior to the candidal infection. LG-pretreated mice received anti-IFN $\gamma$  or diluent 48 h before the i.v.-challenge, and their resistance to disseminated candidiasis was evaluated. The antibody treatment resulted in removal of the protection. MST stands for mean survival times.

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  $\mu$ l or 300  $\mu$ l, 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  $8.4 \pm 2.6$  days and  $9.2 \pm 3.2$  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-IFN $\gamma$ antibody abolishes the protection of LG-treated mice

Effect of anti-IFN $\gamma$  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 IFN $\gamma$  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 of host 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 protective against disseminated candidiasis caused by the same fungus, *C. albicans*, whereas LQ (a glycosidic form of LG) had no protection of mice against disseminated candidiasis. As a matter of the fact, the mice groups treated with LQ, which is lack of induction of Th1 immune response, seemed to succumb to *C. albicans* infection at a faster survival rate than did the diluent alone-received (control) mice groups. If this is the case, once again, it is implicated that the dominance of Th1 immune response induced by the LG can be beneficial in disseminated candidiasis. Now, regarding the absence of protection by the LQ, a possible explanation would be, according to one report [38], permeability rate of LQ (glycoside form of LG) into

tissues is approximately 60 times less than LG when examined by using human intestinal cell line Caco-2. Thus, the poor permeability of LQ may explain why the compound was not protective. On the other hand, in case of daucosterol, we observed that the glycoside form rather than its aglycone showed protective activity in the same model of disseminated candidiasis [14], in which LQ and LG were examined. As a part of conclusion, for the proper immunoregulatory activity, it may be suggested to consider compound permeability. Therefore, in the remainder of the investigation with LQ, we eliminated determining LQ effects in the animal models of disseminated candidiasis.

Next, we determined whether the protective effect is mediated by activation of phagocytic cells, and observed that under an *in vitro* condition LG caused no nitric oxide production from LG-treated RAW 264.7 macrophage/monocyte cells (our unpublished data), which can be one of indications of macrophage activation. This observation implies that the LG had no activating effect of macrophages resulting in no enhancement of phagocytosis. However, upon the administration of LG into mouse body, macrophages could have other way(s) to be activated indirectly by the LG. In fact, in the body, macrophage activity can be enhanced by various activating factors [39,40]. For example, IFN $\gamma$  secreted by activated T cells activates macrophages that may consequently function as an antigen presenting cell (APC) [39,40]. Reminding such aspects, we then focused on examination of LG activity on cellular immune response of Th1 type differentiation based on the cytokine analysis. In our initial determination, LG was found to provoke Th1 type cytokines of IFN $\gamma$  and IL-2 more than IL-4 and IL-10. To examine if this Th1 immune response influences control of *Candida* infection in the host, naïve mice were given LGCD4T, rather than a direct i.p.-injection of LG, before the challenge with the yeast cells and their survival rates were measured. The survival rates of the LGCD4T-reconstituted mice were greater than survival rates of the control mice groups—indicating that the protection was transferable, which lead to a conclusion that aTh1 type immune response by LG might be responsible for the protection.

With the data determined so far, it was not clear whether or not 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 IFN $\gamma$ , 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 the above, macrophage activity might be limited under the circumstance of insufficient amount of IFN $\gamma$ , thus diminishing its APC function and antifungal activity. For the latter case, we even demonstrated that the protection could be abolished by removal of IFN $\gamma$  in the LG-treated mice, but administration of anti-IFN $\gamma$  into LG-treated mice restored the protection. Investigation may 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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