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Original

Inhibitory Effects of *Chlorella* Extract on Airway Hyperresponsiveness and Airway Remodeling in a Murine Model of Asthma

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Abstract: *Chlorella* extract (CE) has been shown to induce production of T helper-1 cytokines, and regulate serum IgE levels in animal models of asthma. We aimed to evaluate whether CE could inhibit ovalbumin (OVA)-induced airway hyperresponsiveness (AHR) and airway remodeling in a murine model of asthma. Balb/c mice were allocated to four groups: a control group (no OVA exposure, not given CE), a CE group (no OVA exposure, given CE), an asthma group (sensitized/challenged with OVA, not given CE) and a CE + asthma group (sensitized/challenged with OVA, given CE). In the asthma and CE + asthma groups, mice were sensitized with OVA on day 0 and day 12, and then challenged with OVA on three consecutive days. In the CE and CE + asthma groups, the mice were given feed containing 2% CE. We assessed AHR to methacholine, and analyzed bronchoalveolar lavage fluid (BALF), serum, lung tissue and spleen cells. Administration of CE was associated with significantly lower AHR in OVA-sensitized and challenged mice. CE administration was also associated with marked reduction of total cells, eosinophils and T helper-2 cytokines (IL-4, IL-5 and IL-13) in BALF. In addition, administration of CE significantly decreased the numbers of periodic acid-Schiff (PAS)-positive cells in OVA-sensitized and challenged mice. Administration of CE also directly suppressed IL-4, IL-5 and IL-13 production in spleen cells of OVA-sensitized and challenged mice. These results indicate that CE can partly prevent AHR and airway remodeling in a murine model of asthma.

Key words: *Chlorella* extract, asthma, Th2 response, probiotics

Introduction

Probiotics are defined as live microorganisms that have beneficial effects on the host by improving the balance of intestinal flora[1]. Recently, many studies have revealed relationships between allergic diseases (such as bronchial asthma, allergic rhinitis, pollinosis and atopic

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dermatitis) and probiotics[2,3]. Since *Chlorella* contains materials that promote the growth of probiotics such as lactobacillus, it is considered a prebiotic.

Chlorella is a genus of single-celled green algae, belonging to the phylum Chlorophyta. It is spherical in shape, about 2–10 μm in diameter, and does not have flagella[4]. *Chlorella* cells contain the green photosynthetic pigment chlorophyll (in their chloroplasts), essential amino acids, carbohydrates, proteins, nucleic acids, minerals, vitamins, dietary fiber, growth factors, and a wide range of antioxidants. *Chlorella* extract (CE) is a popular dietary supplement worldwide, especially in Asia[5].

Previous studies have shown that CE exerts benefits such as enhancing immune function, relieving hypertension, regulating lipid metabolism and tumors, providing antibacterial effects, promoting dioxin excretion, and improving body functions in people with certain illnesses, including two chronic illnesses-fibromyalgia and ulcerative colitis[6,7].

Allergy is defined as a multicellular immune disorder that is characterized by the production of allergen-specific IgE with a predominant T helper-2 (Th2) response[8]. IgE production is promoted by IL-4 from Th2 cells and is suppressed by INF-γ stimulation. Characteristic features of asthma include episodic airflow obstruction, airway inflammation, and airway hyperresponsiveness (AHR, the capacity of the airways to undergo exaggerated narrowing in response to stimuli that do not result in comparable degrees of airway narrowing in healthy subjects). Airway remodeling is associated with severe asthma due to irreversible airway obstruction despite high-intensity treatment.

Lymphocytes, mast cells and eosinophils play important roles in airway inflammation, and studies using targeted deletion approaches have demonstrated that eosinophils are necessary for ovalbumin (OVA)-induced AHR and airway remodeling in mice[9,10]. Airway eosinophilic inflammation is highly regulated by Th2 cytokines IL-4, IL-5 and IL-13, produced by T lymphocytes. Eosinophils can also produce cytokines, chemokines, lipid mediators and growth factors that induce lung fibrosis. Airway remodeling that includes non-reversible structural changes, such as increases in smooth muscle mass, mucus hyperplasia and subepithelial fibrosis, might explain the progressive loss of lung function in patients with asthma[11,12]. Allergic airway inflammation is caused by Th2 cells, mast cells and eosinophils. The Th2 response may be suppressed by activating T helper-1 (Th1) response, to improve the balance between Th1 and Th2 responses.

It has been reported that CE strongly activates Th1 cells and increases the production of INF-γ and IL-2, to strengthen the immune system and host defense[13]. CE has also been shown to enhance intestinal barrier function and suppress IL-5 production in mast cells[14]. Furthermore, CE regulates serum IgE levels and eosinophils in airway epithelium in animal models of asthma[15,16].

In this study, we examined the effect of CE on a murine model of asthma, with a focus on AHR and airway remodeling. This included investigation of whether CE suppresses IgE production.

Materials and methods

Animals

BALB/c mice (female, 6–8 weeks old, weight range 19–24 g) were obtained from the Saitama Experimental Animals Supply Co., Ltd. (Saitama, Japan). All animal experiments were performed under Animal Care and Use Committee approval and conformed to institutional guidelines (Permit Number : 04097).

Mice were allocated to four groups. Mice in the control group were not sensitized or challenged with OVA (they were exposed to saline instead) and were not given CE in their feed. Mice in the CE group were not sensitized or challenged with OVA (they were exposed to saline instead) and were given CE in their feed. Mice in the asthma group were sensitized and challenged with OVA and were not given CE in their feed. Mice in the CE + asthma group were sensitized and challenged with OVA and were given CE in their feed.

CE treatment and OVA-sensitization protocol

BALB/c mice were sensitized with 50 μg/mouse of OVA (Sigma-Aldrich, St Louis, MO, USA) by intra-peritoneal injection on day 0 and day 12, and later challenged intra-nasally with 20 μg/mouse of OVA on three consecutive days (days 25–27) to develop a murine model of asthma. Mice were sacrificed 24 hours after the final OVA challenge. During the 2 weeks before and 4 weeks after the first sensitization with OVA, the mice were given feed containing 2% CE, as described in Fig. 1. The dry feed containing 2% CE was kindly provided by the research laboratories of Chlorella Industry Co. (Fukuoka, Japan).

Measurement of AHR to methacholine

Changes in lung resistance induced by methacholine (MCh) were measured with the Buxco invasive measurement system (Buxco Electronics Inc., Troy, NY, USA) 24 hours after the last OVA challenge. Mice were anesthetized with 80 mg/kg body weight of intra-peritoneal pentobarbital. Tracheas were connected to a ventilator via a 19-gauge needle. The mice were ventilated at a frequency of 150 breaths per minute with 200-μl stroke volumes. Aerosolized saline and increasing concentrations of MCh (dissolved in saline at 6.25 mg/ml, 12.5 mg/ml and 25 mg/ml) were administered. Airflow and pressure changes were recorded with Bio System XA software (Buxco Electronics Inc.).

Bronchoalveolar lavage and cellular analysis

Mice were sacrificed 24 hours after the last OVA challenge. The trachea was exteriorized by blunt dissection, and a small-caliber needle was inserted and secured in the airway. Three successive washes of 0.30 ml saline were instilled and gently aspirated. The recovered bronchoalveolar lavage fluid (BALF) was kept on ice until centrifugation. Each sample of BALF was centrifuged at 300 × g for 7 minutes at 4°C. Cells were washed and resuspended in 100 μl of saline. Total numbers of cells were calculated using a hemocytometer with Turk's

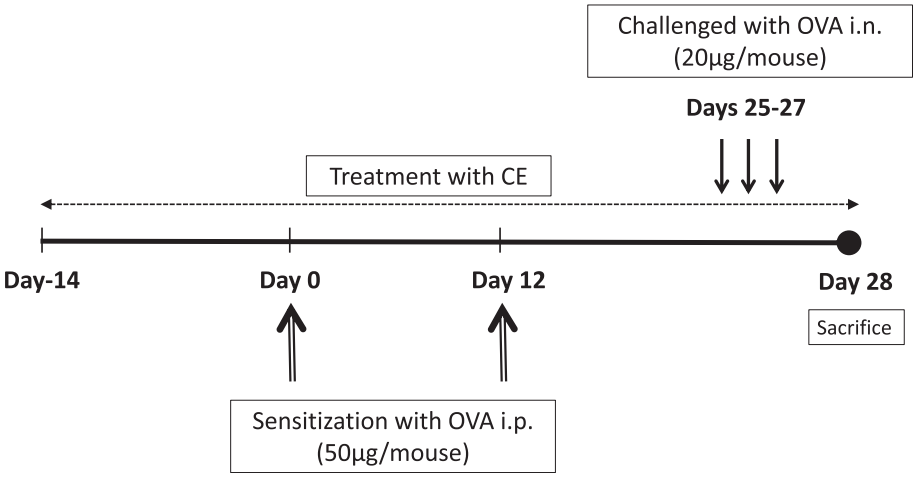

Fig. 1. *Chlorella* extract (CE) treatment and OVA-sensitization protocol
Mice were sensitized with intra-peritoneal (i.p.) injection of OVA (50 μg/mouse) and then challenged intra-nasally (i.n.) with OVA (20 μg/mouse) to develop a murine model of asthma. Mice were administered feed containing 2% CE, beginning 2 weeks before the first sensitization.

solution (Wako, Osaka, Japan). Differential cell counts were performed using cytospin cell preparations (Cytopsin 3; Shandon, Pittsburgh, PA, USA) and stained with Diff-Quick (Dade Behring Inc., Newark, DE, USA). A differential count of 400 cells was performed using standard morphological criteria.

Measurement of cytokine levels in BALF, lung tissue and serum

Levels of cytokines including IL-4, IL-5, IL-13, TARC (a Th2 chemokine) and IFN-γ in BALF and lung tissue, and OVA-specific IgE levels in serum, were measured using ELISA kits (R&D Systems, Minneapolis, MN, USA), by following the manufacturer's instructions. Lung tissue was homogenized in TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and extracted with chloroform. RNA was precipitated with isopropanol, washed with 75% cold ethanol, and resuspended in RNase-free water. Total RNA extracted from whole lung tissue was treated with DNase I (Promega, Madison, WI, USA). Reverse transcription for cDNA synthesis using oligo dT primers was performed with 1 μg of total RNA using a SuperScript II first-strand synthesis system (Invitrogen). Amplification of each cDNA was performed with a*Taq*DNA polymerase (Promega). cDNA was measured by real-time PCR using SYBR Green/ROX master mix (Qiagen, Hilden, Germany) and a C1000 touch thermal cycler (BIORAD, Hercules, CA, USA). The ratio of each mRNA relative to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA was calculated with the $^{\Delta\Delta}$Ct threshold cycle method. Mouse primers for IL-4, IL-5 and IL-13 were obtained from Qiagen.

Histopathological analysis of lung tissue

After performing bronchoalveolar lavage, lungs were dissected from the chest and inflated with

500 μl of 10% formalin in phosphate-buffered saline (PBS) (pH 7.4) for 24 hours, inflated at 10 cm H_2O pressure. Lung tissue samples were dehydrated in ethanol, embedded in paraffin, sectioned (at 5 μm), and stained using the periodic acid-Schiff (PAS) staining system (Sigma-Aldrich, St. Louis, MO, USA). An optical microscope with ×100 and ×400 magnification was used to analyze the lung tissue samples. For analysis of goblet cell hyperplasia, four lung sections were examined per mouse, and percentages of PAS-positive epithelial cells were calculated for four airways from one section. Masson trichrome staining was used to determine collagen deposition in four airways from four different sections per mouse by classification on a 0–4 scale. Semiquantitative analyses were performed by two independent observers who were blinded to information about the four groups.

Cytokine production by spleen cells

Spleen cells isolated from mice in the control, asthma and CE + asthma groups were suspended in RPMI 1640 medium with 10% fetal bovine serum and antibiotics (penicillin, 100 mg/ml ; streptomycin, 100 U/ml) at a concentration of 4.0×10^6 cells/ml. Cells were aliquoted into 48-well plates and stimulated with OVA (100 μg/ml) at 37°C in 5% CO_2. For analysis of cytokine production, cell culture supernatants were collected 72 hours after stimulation with OVA.

Statistical analysis

For comparisons between two groups, we used the unpaired Student's t test. For comparisons of three or more groups, we used one-way ANOVA with Sidak's correction for multiple comparisons. Prism software (GraphPad, La Jolla, CA, USA) was used to perform these analyses. Data are expressed as mean ± SEM, and $P < 0.05$ was considered statistically significant.

Results

Treatment with CE decreased AHR

When we assessed AHR to MCh, there was no difference in airway resistance between mice in the CE group and mice in the control group. AHR to MCh was accelerated in mice in the asthma group compared to mice in the control group. AHR to MCh was significantly inhibited in mice in the CE + asthma group compared to mice in the asthma group (Fig. 2).

Treatment with CE reduced airway inflammation

CE administration did not change the total numbers of cells (Fig. 3a) or the numbers of macrophages, eosinophils and lymphocytes (Fig. 3b) in BALF (control group vs CE group). However, the total numbers of cells (Fig. 3a) and the numbers of eosinophils and lymphocytes (Fig. 3b) in BALF in the CE + asthma group mice were significantly decreased when compared with mice in the asthma group.

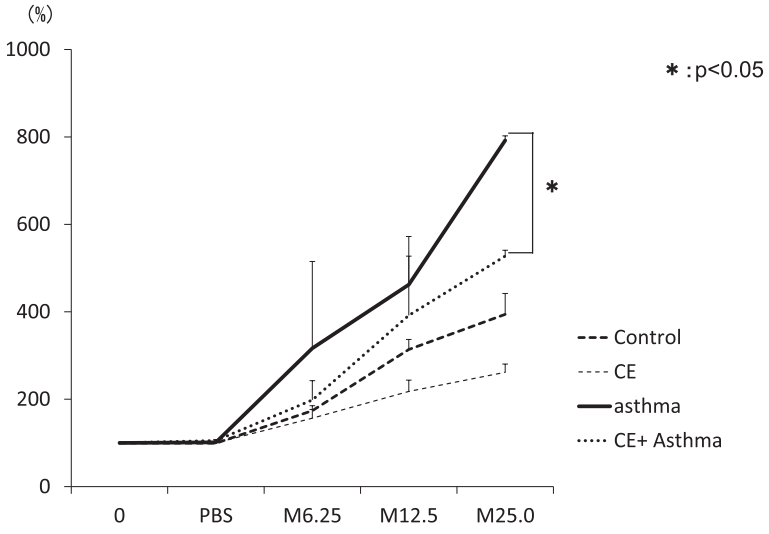

Fig. 2. Treatment with *Chlorella* extract (CE) decreased airway hyperresponsiveness (AHR)

AHR to methacholine in the control group, CE group, asthma group and CE + asthma group (n = 8 for each group, data obtained from two separate experiments), expressed as percentage change from baseline level of lung resistance. Results are mean values, with error bars representing SEM.

Treatment with CE reduced cytokine levels in BALF and lung tissue

IL-4 and TARC levels were significantly decreased in BALF of CE + asthma group mice when compared with asthma group mice (Fig. 3c). However, there were no differences in INF-γ levels in BALF between the groups (Fig. 3c). Although IL-5 and IL-13 could not be detected in BALF by ELISA (data not shown), the mRNA levels of IL-4, IL-5 and IL-13 in lung tissue were decreased in mice in the CE + asthma group when compared with mice in the asthma group (Fig. 3d).

Treatment with CE reduced OVA-specific IgE levels in serum

Serum levels of OVA-specific IgE were significantly increased in mice in the asthma group when compared with mice in the control and CE groups, and this increase was significantly smaller in the CE + asthma group (Fig. 4).

Treatment with CE reduced airway remodeling and airway fibrosis

Mice in the asthma group had an increase in the percentage of PAS-positive airway epithelial cells compared with those in the control group (Fig. 5a). Treatment with CE significantly reduced the percentage of PAS-positive airway epithelial cells induced by OVA challenge (Fig. 5a). When airway fibrosis was studied by treating tissue with Masson trichrome stain, the increase in airway fibrosis by OVA was significantly inhibited by treatment with CE (Fig. 5b).

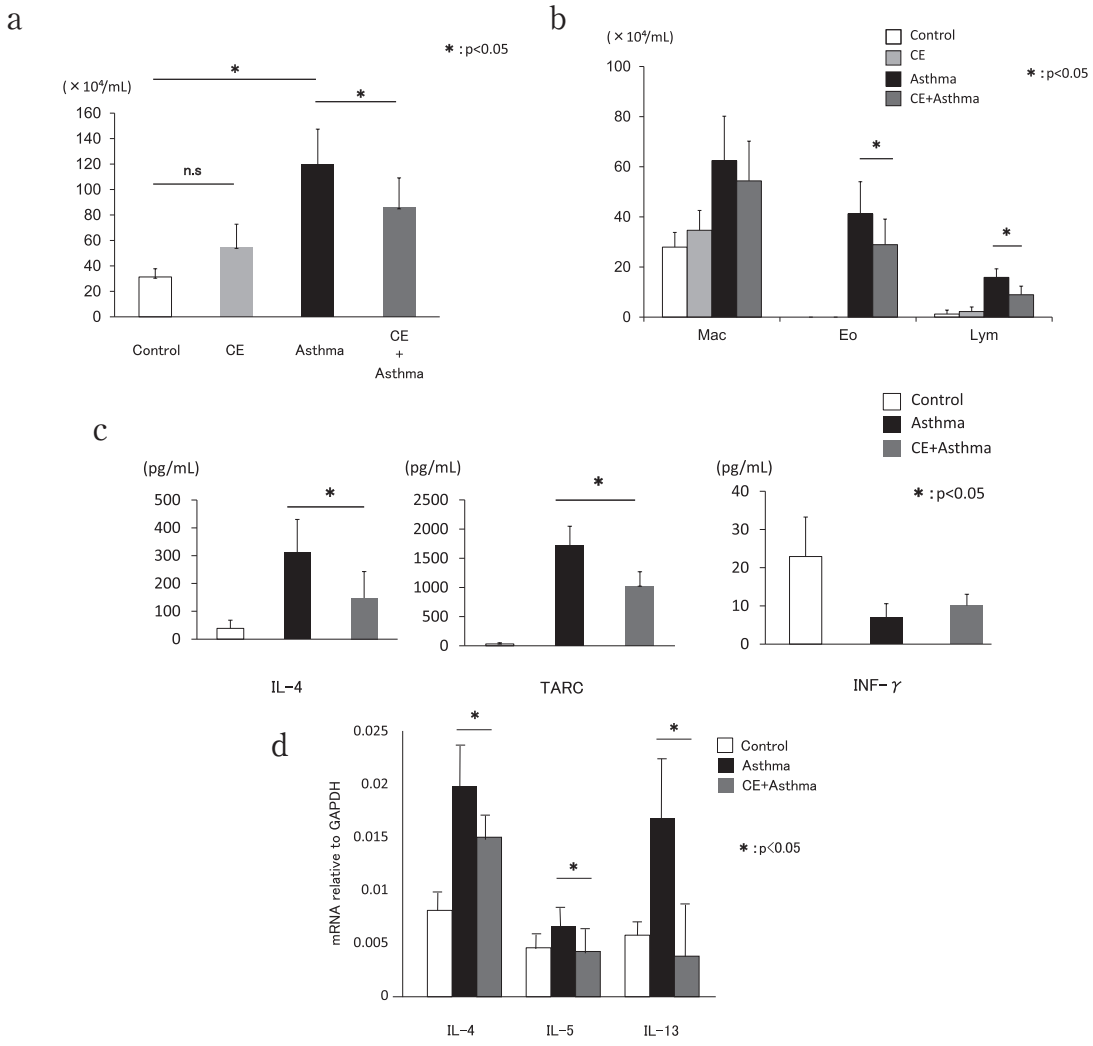

Fig. 3. Treatment with CE reduced cytokine levels
Analysis of inflammatory cells and cytokines in BALF. (a) Total numbers of inflammatory cells in BALF; n.s = not significant. (b) Numbers of macrophages (Mac), eosinophils (Eo) and lymphocytes (Lym) in BALF. (c) Cytokine levels in BALF measured by ELISA. (d) Cytokine levels in lung tissue measured by ELISA. Results are mean values, with error bars representing SEM (n = 8 for each group).

Treatment with CE inhibited cytokine production by spleen cells

In spleen cells of mice in the asthma group, CE did not increase INF-γ expression (data not shown), but CE significantly inhibited OVA-stimulated production of Th2 cytokines IL-4, IL-5 and IL-13 (Fig. 6).

Discussion

We examined the effects of CE in a mouse model of OVA-induced asthma. We demonstrated that CE suppressed AHR induced by MCh, and signs of airway inflammation-total numbers of cells, and numbers of eosinophils and lymphocytes in BALF. In addition, CE suppressed airway smooth muscle hypertrophy and goblet cell hyperplasia, which are features of airway remodeling

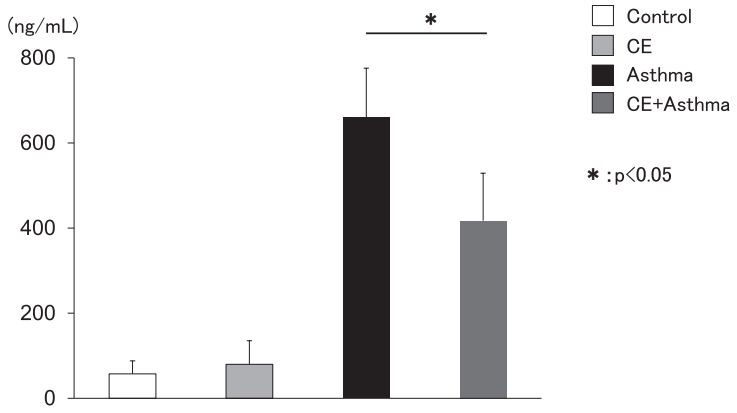

Fig. 4. Treatment with CE reduced OVA-specific IgE levels in serum
Analysis of serum OVA-specific IgE level in the control group, CE group, asthma group and CE + asthma group. Results are mean values, with error bars representing SEM.

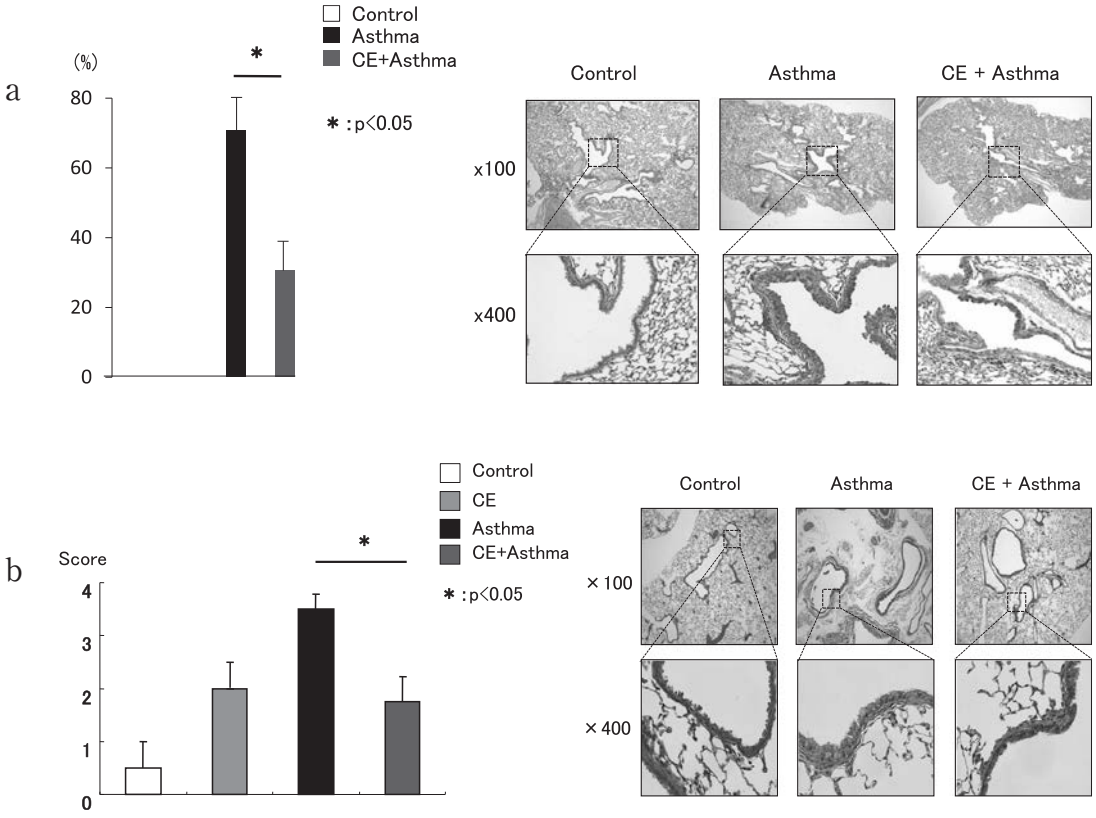

Fig. 5. Treatment with CE reduced airway remodeling and airway fibrosis
(a) Percentage of goblet cell hyperplasia for the control, asthma and CE + asthma groups (n = 8 for each group), and representative lung sections showing PAS staining for the control, asthma and CE + asthma groups at ×100 and ×400 magnification.
(b) Scoring of airway fibrosis for the control, CE, asthma and CE + asthma groups (n = 8 for each group), and representative lung sections showing Masson trichrome staining for the control, asthma and CE + asthma groups at ×100 and ×400 magnification. Graphed results are mean values, with error bars representing SEM. The control group exhibited minimal epithelial PAS and Masson trichrome staining. In contrast, the asthma group showed enhanced epithelial PAS and Masson trichrome staining, which were significantly inhibited by CE treatment.

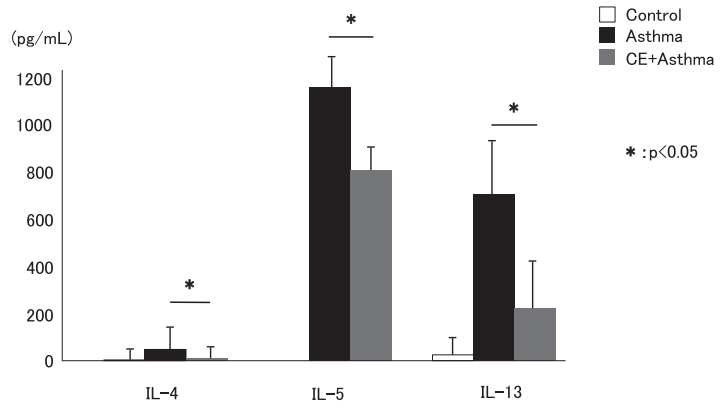

Fig. 6. Treatment with CE inhibited cytokine production by spleen cells. Analysis of IL-4, IL-5 and IL-13 in the supernatant after stimulation of spleen cells with OVA in the control, asthma and CE + asthma groups (n = 8 for each group). Results are mean values, with error bars representing SEM.

in asthma. To our knowledge, this is the first report on the inhibitory effects of CE on airway remodeling in a mouse model of asthma. As Th2 cytokines can directly induce airway smooth muscle hypertrophy and goblet cell hyperplasia, CE is likely to inhibit airway smooth muscle hypertrophy and goblet cell hyperplasia at least partly by suppressing Th2 cytokines.

Chlorella supplements, which contain a variety of substances, are sold as health supplements in East Asian countries, including Japan. Reports on the health benefits of *Chlorella* include its use in a wide variety of diseases, ranging from depressive disorders to breast cancer[17, 18]. However, the detailed mechanisms of action for *Chlorella*'s effects are unknown. A previous report showed that oral administration of CE enhanced the resistance of mice to *Listeria monocytogenes* infection owing to enhancement of Th1 response[19]. It has also been reported that CE prevents the release of allergic mediators by suppressing calcium uptake by mast cells, resulting in inhibition of immediate-type allergic reactions[20]. In addition, polysaccharide-rich components of *Chlorella* pyrenoidosa have been shown to induce IL-1β and TNF-α production by macrophages. These data suggest that *Chlorella* exerts its effects by shifting the Th1/Th2 balance to Th1 dominance.

Allergic diseases, including asthma, are considered to be Th2 dominant, and inhibition of Th2 cytokines has been shown to be effective in treating some allergic diseases in humans. Mepolizumab, an anti-IL-5 monoclonal antibody, has dramatic effects on patients with severe asthma[21]. Dupilmab, a human anti-IL-4 receptor α monoclonal antibody, has been shown to improve skin condition in patients with atopic dermatitis[22]. In our study, we showed that administration of CE decreased the number of eosinophils in BALF in a murine model of asthma. This inhibition could have resulted from a CE-induced decrease in Th2 cytokines. We also showed that administration of CE resulted in a significant decrease in serum IgE levels, which is in line

with previous data from mice[16].

A previous report has shown that endotoxin levels in CE are below analytical detection limits[23]. However, CE has been shown to produce lipopolysaccharide-like substances that contribute to the Th1 response[24]. Also, CE has been shown to enhance IFN-γ production in *Listeria monocytogenes*-infected mice[19], casein-injected mice[16] and OVA-immunized mice[20]. However, in a mouse model of *Der f*-induced atopic dermatitis, it has been shown that CE reduces eosinophilic inflammation, IL-4 expression and IFN-γ expression in skin[25]. In our study, CE did not lead to an increase in IFN-γ in BALF, indicating that IFN-γ did not play a critical role in CE-induced inhibition of airway inflammation and AHR.

To our knowledge, no previous studies have investigated the effects of CE on AHR in an animal model of asthma. However, one study has looked at the effects of CE in chronic obstructive pulmonary disease (COPD). That study showed that CE did not change forced vital capacity (FVC), forced expiratory volume in the first second (FEV1) or FEV1/FVC in patients with COPD[26], suggesting that CE does not have direct bronchodilating effects. A marked difference between COPD and asthma is the type of airway inflammation; it is neutrophilic in COPD and eosinophilic in asthma. This might explain why no bronchodilating effect of CE was seen in COPD, while AHR was reduced by CE in a model of asthma.

A previous report indicated that CE induced maturation of dendritic cells (DCs), and that the resulting mature DCs activated naive T cells and stimulated T cell proliferation and IFN-γ secretion[27]. It has also been reported that CE is involved in activation of toll-like receptor 2 (TLR2)[28] and enhances Th1 cytokine production. Furthermore, it has been reported that TLR2 induces regulatory T cells (Tregs), which results in suppression of asthma manifestations in mice[29]. Since TLR2 in macrophages and DCs produces inflammatory cytokines, mature DCs induced by CE may enhance Tregs via activation of TLR2 and result in a shift of Th1/Th2 balance to Th1 dominance. *Chlorella* containing lipopolysaccharide analogs have a powerful ability to lead Th1 response[25]. CE therefore has the potential to enhance Th1 cytokine production.

We examined the direct action of CE using spleen cells. Although INF-γ was not increased by the addition of CE to spleen cells (data not shown), CE suppressed production of Th2 cytokines IL-4, IL-5 and IL-13. These results suggest that *Chlorella* may directly act on T cells at a cellular level. Although several mechanisms by which CE could change the balance of Th1 and Th2 response have been proposed, the details are unclear and further investigation is needed.

Th2 cytokines are mainly produced by Th2 cells, but they are also produced by mast cells, basophils and type 2 innate lymphoid cells (ILC2s)[30]. We demonstrated that CE inhibits the production of Th2 cytokines by murine spleen cells. This suggests that CE directly suppresses the production of Th2 cytokines by Th2 cells. It has been reported that CE suppresses IL-5 production by mast cells[14]. Therefore, CE may suppress Th2 cytokine production not only by Th2 cells but also by mast cells. ILC2s play an important role in allergen-independent Th2-induced inflammation caused by production of IL-5 and IL-13. However, the effect of CE on

ILC2s is still unknown.

In conclusion, we observed that CE treatment in an allergic mouse model suppressed allergic inflammation and AHR, the main reaction of bronchial asthma, by inhibiting the Th2 response. CE also directly suppressed Th2 response on immune cells including spleen cells. These results suggest that CE may protect against the development of allergic asthma due to repeated allergen exposure.

Acknowledgements

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Conflict of interest disclosure

The authors have no conflicts of interest to disclose.

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