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ORIGINAL ARTICLE

Naringenin Chalcone Suppresses Allergic Asthma by Inhibiting the Type-2 Function of CD4 T Cells

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ABSTRACT

Background: Some polyphenols possess anti-allergic activities. Naringenin chalcone is one of the polyphenols that is present in the skin of red tomatoes. In this study, we investigated the effect of naringenin chalcone in allergic responses *in vivo* using an experimental mouse model system of allergic asthma.

Methods: Allergic airway inflammation was induced in mice by sensitization and challenge with ovalbumin. Naringenin chalcone was orally administrated every day during the course of the experiment. Airway hyperreactivity, the eosinophilic infiltration in the bronchioalveolar lavage fluid and Th2 cytokine production from splenic CD4 T cells were assessed.

Results: Eosinophilic airway inflammation, airway hyperreactivity and Th2 cytokine production from CD4 T cells were significantly suppressed in mice that were treated with naringenin chalcone. Hyperproduction of mucus was slightly reduced.

Conclusions: The results of this study suggest that naringenin chalcone suppresses asthmatic symptoms by inhibiting Th2 cytokine production from CD4 T cells. Thus, naringenin chalcone may be a useful supplement for the suppression of allergic symptoms in humans.

KEY WORDS

airway hyperreactivity, asthma, prevention, Th2 cell, tomato extract

INTRODUCTION

The number of allergic patients has increased in developed countries.[1] Asthma is a chronic inflammatory disease of the lower airways that causes airway hyperreactivity (AHR) to a wide variety of specific and non-specific stimuli.[2] In most cases, the strength of AHR correlates with the level of airway inflammation, another hallmark of asthma, which is characterized by eosinophil infiltration, mucus hyperproduction, production of Th2 cytokines (IL-4, -5 and -13) and allergen-specific IgE.[3-5]

Th2 cells that produce these cytokines play an important role in allergic asthma.[6] IL-4 and IL-13 produced from Th2 cells initiate the synthesis of antigen-specific IgE from B cells. IgE antibodies circulate in the blood and bind to high affinity IgE receptors (FcεRI) on the surface of mast cells. When allergens are bound to the receptor-bound IgE molecules, mast cells become activated and release cytokines, chemokines, and chemical mediators, such as histamine, prostaglandin, and leukotrienes, which induce airway smooth-muscle contraction and increased vascular permeability.[6,7] In the epithelial cells, IL-4 and IL-13 can induce goblet cell hyperplasia.[8]

Topical corticosteroids and topical immunosuppressants are commonly used for the treatment of allergic diseases. However, these drugs strongly inhibit the immune system, and sometimes induce several side effects, particularly when they are used for extended periods. Therefore, substitutes for steroids are still required. Some polyphenols possess anti-allergic activities.[9-13] Tomatoes are a popular vegetable that are produced almost all over the world. Tomatoes contain many polyphenols, such as chlorogenic acid and rutin. Naringenin chalcone (NGC) is

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one of the polyphenols that is present in the skin of red tomatoes.[10] Therefore, we investigated whether the *in vivo* administration of NGC induces any suppressive activities on allergic reactions, using a popular experimental mouse model system of allergic asthma.

METHODS

ANIMALS

BALB/c mice were purchased from Clea Inc., Tokyo, Japan. The mice used in this study were 6 to 7 weeks of age. A total of three independent experiments were performed for each experiment (n = 5 per group). All of the mice used in this study were maintained under specific pathogen free conditions. All of the animal care was conducted in accordance with the guidelines of Chiba University.

SAMPLE PREPARATION FROM THE TOMATO SKIN EXTRACT

The tomato (*Lycopersicon esculentum* Miller) skin extract containing polyphenols was prepared by incubating a mixture of tomato skin with 60% (v/v) ethanol at 60℃ for 2 hours, followed by lyophilization. The extract contained approximately 0.2% naringenin chalcone (NGC). NGC was separated from the tomato skin extract by a HPLC method using a Capcell Pak C18 column (250 mm × 15 mm I.D.; Shiseido, Tokyo, Japan). The elution was carried out with acetonitrile/water (33 : 67, v/v) mixture containing 0.1% trifluoroacetic acid at a flow rate of 5 mL/minute, at room temperature with the instrument's wavelength set at 350 nm for the monitoring of the eluant. The peak fraction was collected, concentrated under reduced pressure to remove the organic solvents, and then freeze-dried. The purity of NGC was >98 %.

INDUCTION OF ALLERGIC ASTHMA AND THE ADMINISTRATION OF NARINGENIN CHALCONE

The mice were sensitized by intraperitoneal injection of 100 μg Ovalbumin (OVA: SIGMA-Aldrich, St. Louis, USA) adsorbed to 1 mg alum (PIERCE, Rockford, USA) on day 0 and day 7. The mice were then challenged with aerosolized OVA in saline (10 mg/mL) by inhalation for 30 minutes using a supersonic nebulizer (model NE-U07; Omuron Co., Tokyo, Japan) on day 21 and 23. NGC (0.8 mg/kg) was orally administered in a 0.5% carboxymethyl cellulose sodium salt solution (5 mL/kg of body weight) every day during the entire course of the experiments. A 0.5% carboxymethyl cellulose sodium salt solution without NGC was administrated as a placebo. The half-life of the metabolites of NCG in the blood of rat receiving the equivalent amount of NCG was 5.5 ± 1.7 hours,[14] and about 60% of the NCG appeared to be absorbed.

MEASUREMENT OF AIRWAY HYPERREACTIVITY (AHR)

The AHR was assessed by methacholine-induced airflow obstruction 24 hours after the last antigen challenge as previously described.[15] The respiratory parameters were obtained by exposure of the mice to 0.9% saline mist, followed by incremental doses of aerosolized methacholine (0, 12, 24, 48 mg/mL in a saline solution). The AHR was assessed by a computer-controlled small animal ventilator (SCIREQ, Montreal, Canada).

COLLECTION OF THE BRONCHIOALVEOLAR LAVAGE FLUID

Bronchioalveolar lavage (BAL) was performed 24 hours after the last OVA challenge as described previously.[16] All the BAL fluid was collected and the cells were counted in the 100-μl aliquots. A total of 1×10^5 viable BAL cells were cytocentrifuged onto slides by a Cytospin4 (Thermo Electron, Waltham, USA) and stained with May-Grunwald-Giemza solution (MERCK, Darmstadt, Germany). A total of 500 leukocytes were counted for each slide. The cell types were identified using morphological criteria. The percentages of each cell type were calculated.

MEASUREMENT OF THE ANTIBODY TITER IN SERUM

The concentration of OVA-specific IgM, IgG1, IgG2a and total IgE antibodies in serum was determined by ELISA as previously described.[17]

LUNG HISTOLOGY

The mice were sacrificed by asphyxiation at 48 hours after the last OVA challenge, and lungs were infused with 10% (v/v) formalin in PBS buffer for fixation. The lung samples were sectioned, stained with hematoxylin and eosin (H&E) reagents or with periodic acid-Schiff (PAS) reagent, and examined for pathological changes under a light microscope at ×100 magnification. The number of infiltrated mononuclear cells in the peribronchiolar regions was calculated by direct counting in four different fields per slide.

QUANTITATIVE PCR ANALYSIS

Total RNA was isolated from lungs (3 mice in each group) using the TRIzol reagent (SIGMA-Aldrich, St. Louis, USA). Reverse transcription was carried out with Superscript II RT (Invitrogen, Carlsbad, USA). The samples were then subjected to real-time PCR analysis on an ABI PRISM 7300 Sequence Detection System (Applied Biosystems, Foster City, USA) using standard conditions. The primers and the TaqMan probes for the detection of Muc5ac, gob-5, and hprt were purchased from Applied Biosystems. The expression of mRNA was normalized using the hprt signal.

CELL PREPARATION AND THE MEASUREMENT OF CYTOKINES

The mice treated with or without NGC were sacrificed after OVA inhalation. The single-cell suspensions of the spleen cells were prepared in RPMI 1640 medium. Splenic CD4 T cells were purified by magnetic beads and an Auto-Macs Sorter (Miltenyi Biotech, Gladbach, Germany). The purity of CD4 T cells was >90%. We thereafter cultured CD4 T cell (4×10^5) and irradiated (3000 rad) normal BALB/c splenocytes (1×10^6) with OVA (100 μg/mL) for indicated hours. The supernatants were collected three or four days later. The concentrations of IL-2, IL-4, IL-5, IL-13, and IFN-γ were measured by ELISA as described previously.[18]

DATA ANALYSIS

The statistical analysis was performed using the two-tailed Student's *t*-test. All values are expressed as the mean ± standard deviation (SD).

RESULTS

NGC PREPARED FROM THE TOMATO EXTRACT REDUCES EOSINOPHILIC INFILTRATION IN THE BAL FLUID AND THE AHR

The aim of this study was to examine the effect of oral administration of NGC on allergic asthma. In order to induce the allergic asthma, the mice were sensitized with OVA-alum on days 0 and 7, and then challenged with aerosolized OVA on days 21 and 23. The NGC was orally administered every day until the day of sacrifice. Firstly, the development of AHR was examined by measuring the methacholine induced airflow obstruction using a mechanical ventilator (Fig. 1 A). The extent of AHR in the mice that were treated with NGC was attenuated in comparison with the placebo control group. At this time, the BAL fluid was harvested and examined for the infiltration of the inflammatory cells (Fig. 1B). The absolute numbers of eosinophils, neutrophils, lymphocytes and macrophages were determined based on the morphological criteria. As shown in Figure 1B, total cell numbers of the infiltrating leukocyte decreased significantly in the NGC-treated mice. A significant decrease in the absolute number of eosinophils was also observed. The results suggest that the OVA-induced AHR and the airway inflammation were attenuated by the oral administration of NGC. We also measured antibody titers in the serum of these mice one day after the last inhalation. The concentration of OVA-specific IgM, IgG1 and IgG2a and total IgE in the serum of NGC-treated mice was comparable to that of placebo control mice (Fig. 1C).

HISTOLOGICAL ANALYSIS OF LUNGS FROM THE NGC-TREATED MICE

The histological changes in the lungs of OVA-sensitized and OVA-challenged NGC-treated mice were examined. An inflammatory cell infiltration was not found in the lungs of the mice that did not receive either the OVA sensitization or the challenge (Fig. 2 A, No treat). After the OVA challenge, a substantial number of mononuclear cells were infiltrated in the peribronchiolar regions in the OVA-sensitized mice treated with placebo (Fig. 2A, Placebo). The infiltration was moderate in the mice that received NGC in comparison with the mice that were on placebo (Fig. 2A, NGC). The number of the infiltrated mononuclear cells was assessed semi-quantitatively, and decreased in the NGC-treated mice (Fig. 2B).

We then examined the levels of mucus hyperproduction by PAS staining. The representative staining profiles of the bronchiolar region of the asthmatic lung are shown in Figure 2C. A specific staining was not detected in the mice that did not receive the OVA-sensitization and the OVA-challenge (Fig. 2C, No treat). A strong PAS staining was found in the bronchioles of the asthmatic lung of the mice treated with the placebo, whereas the staining levels appeared to be lower in the mice treated with NGC (Figure 2C, comparing placebo and NGC). Consequently, we examined the expression of Muc5ac and gob-5, and slightly decreased expression was found in the lungs of the NGC-treated mice (Fig. 2D). These results suggested that the level of mucus hyperproduction was reduced moderately after the oral administration of NGC.

NGC REDUCES TH2 CYTOKINE PRODUCTION FROM SPLENIC CD4 T CELLS

In order to assess the effect of NGC on the effector function of the CD4 T cells, cytokine production in the splenic CD4 T cells from NGC-treated mice was examined (Fig. 3). The splenic CD4 T cells were purified from the OVA-sensitized and -challenged mice treated with or without NGC, and were stimulated subsequently with OVA and irradiated APCs *in vitro*. Three and four days later, culture supernatants were collected, and the concentration of cytokines (IL-4, IL-5, IL-13, IFN-γ and IL-2) were determined by ELISA. The production of IL-4, IL-5, and IL-13 was lower in the mice treated with NGC in comparison with the mice treated with placebo. In contrast, the levels of IL-2 and IFN-γ were comparable. These results indicate that the oral administration of NGC resulted in reduced Th2 cytokine production from CD4 T cells of the spleen.

DISCUSSION

The administration of tomato extract has been reported to improve perennial allergic rhinitis in humans.[12] In this report, we used NGC that was purified from the skin of red tomatoes, and demonstrated that the oral administration of NGC suppressed airway inflammation and the AHR in a mouse allergic asthma model. NGC appears to suppress the Th2-dependent

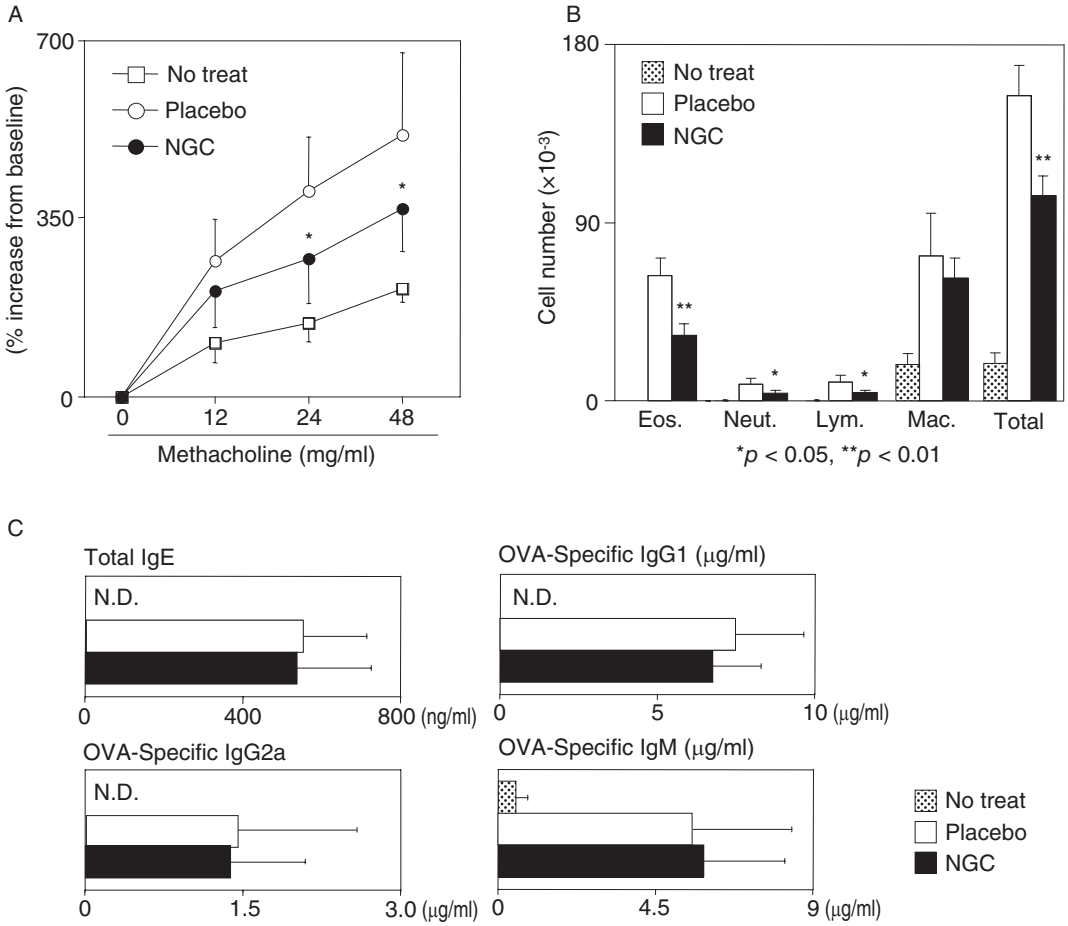

Fig. 1 Reduced airway hyperresponsiveness (AHR) and airway inflammation in NGC-treated mice. AHR and airway inflammation were induced with OVA-sensitization and OVA-challenge in the mice treated with or without NGC. (**A**) One day after the last OVA challenge, AHR in response to increasing doses of methacholine was assessed by measuring lung resistance. (**B**) The absolute numbers of eosinophils (Eos.), neutrophils (Neut.), lymphocytes (Lym.) and macrophages (Mac.) in the BAL fluid are shown. The results were obtained using the values from cell counting, the percentages of the cells, the total cell number per milliliter, and the volume of BAL fluid recovered. The samples were collected 24 hours after the last OVA challenge. (**C**) The levels of total IgE and OVA-specific IgG1, OVA-specific IgG2a and OVA-specific IgM in the serum were determined by ELISA. The samples were collected 24 hours after the last OVA challenge. Four independent experiments were done with similar results. Five animals from each group were individually examined, and the mean values with their SD are indicated. The differences were statistically significant between the mice treated with placebo and the mice treated with NGC (*$p < 0.05$, and **$p < 0.01$).

airway inflammatory responses via the inhibition of the Th2 cytokine production from CD4 T cells of the spleen.

Naïve CD4 T cells differentiate into effector T cells upon stimulation by antigen. The cytokines present in the environment of the antigen- recognition by T cell receptor on CD4 T cells determine which effector T cells are induced such as Th1, Th2 or Th17 cells.[19] In this OVA induced-allergic airway inflammation model, Th2 cells are induced by sensitization with OVA and Alum injection.[20] We examined the cytokine production of CD4 T cells in the NGC-treated mice. As a result, the Th2 cytokine production in CD4 T cells of the NGC-treated mice was lower than that of placebo-treated mice (Fig. 3). We investigated the proliferation of CD4 T cells from the NGC-treated or untreated mice by measuring thymidine-uptake, and found that there were no significant differences in proliferative responses (data not shown). These results suggest that NGC suppresses the Th2 cytokine production from CD4 T cells without affecting the proliferative ability of CD4 T cells.

Late phase allergic responses in the airway are induced by Th2 cells and by eosinophills that infiltrate

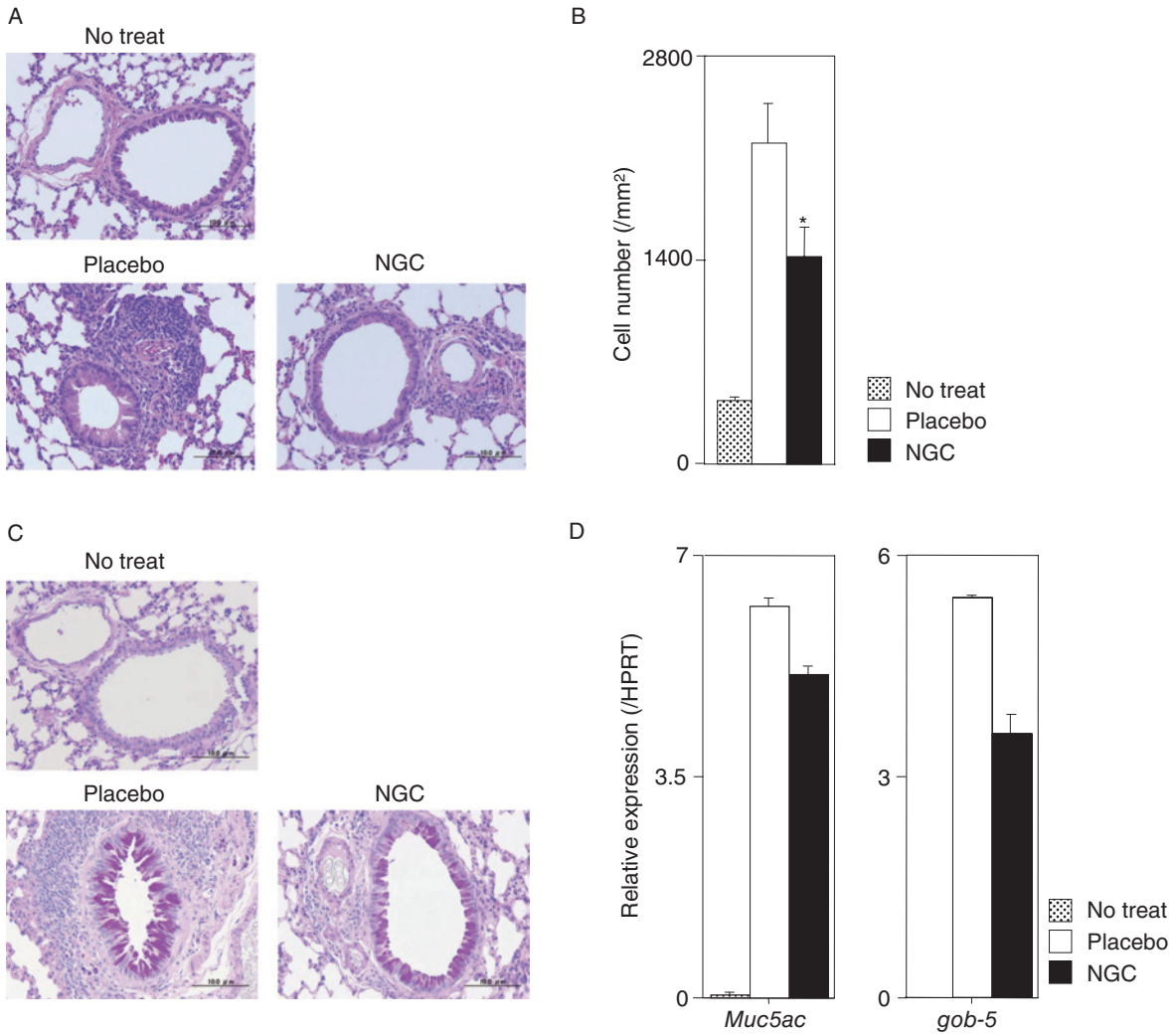

Fig. 2 Reduced leukocyte infiltration into the lungs and mucus production in NGC-treated mice. The levels of OVA-induced airway inflammation and mucus production in the mice treated with NGC were examined by histological analysis and quantitative real-time RT-PCR. (**A**) Antigen-induced leukocyte infiltration into the lungs was evaluated using hematoxylin and eosin (H&E) staining. (**B**) The number of infiltrated mononuclear cells in the perivascular and peribronchiolar regions was calculated by direct counting from four different fields per slide. The differences were statistically significant between the mice treated with placebo and the mice treated with NGC (*$p < 0.05$). (**C**) Antigen-induced goblet cell hyperplasia was evaluated by PAS staining. The representative photographic views of the mice treated with or without NGC are shown. (**D**) The total mRNA was prepared from the lung of the mice treated with or without NGC, and the mRNA levels of Muc5ac and gob-5 were examined. The data represent the mean values of Muc5ac and gob-5 mRNA expression normalized with *hprt* expression. The mean values with the standard deviation ($n = 5$) are shown. Three independent experiments were performed with similar results.

in the lung from 2 to 6 hours after antigen exposure.[6] In this study, NGC administration inhibited the production of IL-4, IL-5 and IL-13 from CD4 T cells. IL-13 is known to induce AHR in the absence of inflammatory cells.[21] IL-13 and IL-4 also play an important role in the production of mucus.[5] These symptoms are characteristic of asthma, and are linked to asthma fatality. IL-5 is known to activate and enhance the survival of eosinophils.[22] Regarding the proteins present in eosinophils, the most prominent granule protein is a cationic heme-containing protein known as eosinophil peroxidase (EPO). The second is the major basic protein (MBP), which is also released during eosinophil degranulation. Although these eosinophil granules induce cytotoxicity in lung tissue such as airway epithelial cells, it has also been reported that EPO and MBP do not contribute to allergen-induced airway pathogenesis in mice.[23,24] In any event, the reduction of Th2 cytokine production from CD4 T cells may result in attenuated eosinophillic inflammation

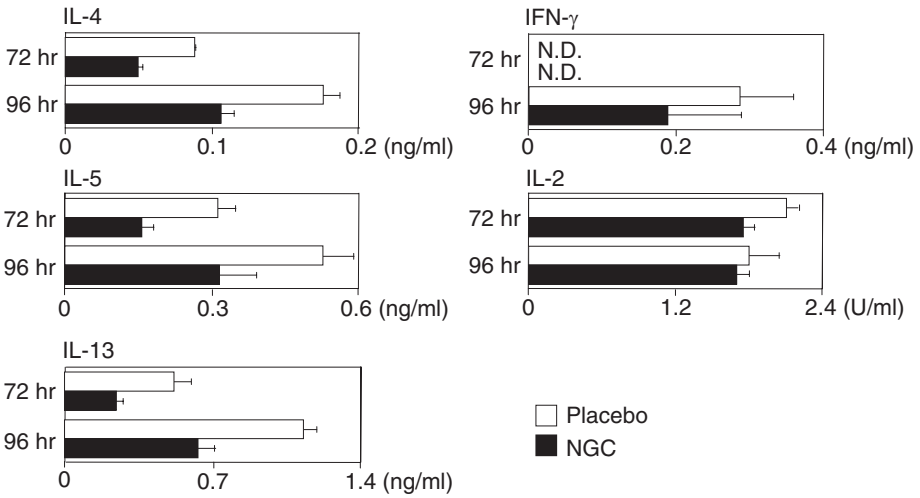

Fig. 3 Reduced Th2 cytokine production from splenic CD4 T cells in mice treated with NGC. Splenic CD4 T cells from the NGC-treated mice or the placebo-treated mice were prepared and stimulated subsequently *in vitro* with OVA and APCs. The culture supernatants were collected 72 and 96 hours later. The mean values with standard deviation are shown ($n = 5$). Three independent experiments were performed with similar results. The differences were statistically significant between the mice treated with placebo and the mice treated with NGC. N.D., not detectable.

and AHR in NGC-treated mice.

IgE and mast cells play a crucial role in the induction of acute phase allergic responses that occur immediately after antigen exposure.[6] The levels of IgE were unchanged by treatment with NGC (Fig. 1C). It is well established that IL-4 is produced by Th2 cells. However, there are other pathways which induce IgE production in allergic inflammation. IL-4 and IL-13 are secreted not only by CD4 T cells but also by mast cells, and IL-4 from mast cells influences the production of IgE from B cells.[25] In addition, soluble CD23 regulates the production of IgE from B cells.[26,27] NGC may not suppress the production of IgE through these pathways, although the reduction of Th2 cytokines from CD4 T cells was detected. We have not detected any defects in B cell function in mice with NCG administration (unpublished observation).

We previously reported that NGC inhibits histamine release from mast cells.[10] Therefore, although NGC treatment did not inhibit IgE production, NGC could suppress the type I allergic reaction by inhibiting the release of histamine from mast cells. The mechanisms by which NGC suppresses Th2 cytokine production of CD4 T cells and deregulation from mast cells are not clearly understood. Tan *et al*. reported that *trans*-Resveratol, a polyphenol of red wine, inhibited human eosinophil activation and deregulation via inhibition of activation of p38 and ERK 1/2.[28] Therefore, NGC is a polyphenol from tomatoes and it may inhibit some signaling molecules in CD4 T cell and mast cells. Further investigation is required to elucidate the mechanism by which NGC reduces the Th2 cytokine production from CD4 T cells or the release of chemical mediators from mast cells.

We administered 0.8 mg/kg/day NGC. NGC is contained in *tomato cultivar for processing* or *cherry tomato* but not in a commonly consumed tomato in Japan. Fifteen cherry tomatoes contain approximately 0.8 mg NGC, and thus the amount of NGC which is taken by a mouse (20 g) per day is equivalent to 0.3 of the amount contained in one cherry tomato. Humans (60 kg) may therefore need to eat 900 cherry tomatoes every day for 3 weeks to consume the necessary amount. As a result, we need to purify NGC from tomatoes and provide it as a supplement for practical administration.

In conclusion, the oral administration of NGC was shown to suppress Th2 cytokine production from CD4 T cells in the spleen and to attenuate allergic airway inflammation and AHR. The high-dose, long-term oral administration of the tomato extract containing NGC did not show any severe adverse events.[29] Therefore, taking NGC as a supplement may be beneficial to patients with allergies.

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