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마우스 수지상세포와 CD4+ T cell의 상호작용에 미치는 예쁜꼬마선충 조항원의 효과

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ABSTRACT

Helminth infections have been known to modulate the immune response of the host and several helminth-derived molecules were suggested to have protective effects against allergic diseases and autoimmune diseases. In the previous study, crude extracts of *Caenorhabditis elegans* (CEC) have been shown to suppress airway inflammation in a murine asthma model. The present study was undertaken to investigate the effects of CEC on the interaction between bone marrow-derived dendritic cells (BMDCs) and CD4+ T helper cells of mice. CEC treatment on BMDCs markedly attenuated the expression of MHC class II molecules and co-stimulatory molecules including CD80, CD86, and CD40 compared to BMDCs stimulated by LPS alone (P<0.01). Production of pro-inflammatory cytokines including IL-12p70, IL-6 and TNF-α, was significantly decreased and that of an anti-inflammatory cytokine, IL-10, was elevated by stimulation of BMDCs with CEC and LPS than those stimulated by LPS alone (P<0.05). Moreover, LPS+CEC-pulsed BMDCs suppressed proliferation of CD4+ T cells. CD4+ T cells cultured with LPS+CEC-pulsed BMDCs produced significantly higher amounts of Th1 cytokine, interferon-γ (IFN-γ), and lower Th2 cytokines including IL-4, IL-5 and IL-13 compared with those with LPS-pulsed BMDCs (P<0.05). Taken together, the present results suggest that CEC activates BMDCs and induces naïve CD4+ T cells to differentiate into Th1 cells and also suppresses the proliferation of CD4+ T cells. Therefore, CEC may

modulate the immune response of hosts by interacting with BMDCs, resulting in the differentiation of naïve T cells to Th1 cells.

Keywords: ***Caenorhabditis elegans*****, crude extracts, dendritic cells, CD4+ T helper cells, immune regulation**

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LIST OF ABBREVIATIONS

BMDCs: Bone marrow-derived dendritic cells

APC: Antigen presenting cells

MHC: Major histocompatibility complex

CD: Cluster of differentiation

BAL fluid: Bronchoalveolar lavage fluid

LPS: Lipopolysaccharide

CEC: Crude extracts of *Caenorhabditis elegans*

FBS: Fetal bovine serum

PBS: Phosphate buffered saline

GM-CSF: Granulocyte macrophage colony-stimulating factor

ELISA: Enzyme-linked immunosorbent assay

IL: Interleukin

IFN-γ: Interferon-gamma

TNF-α: Tumor necrosis factor-alpha

TGF-β: Transforming growth factor-beta

INTRODUCTION

Asthma is characterized by airway hyper-responsiveness, chronic airway inflammation, increased mucus production, and airway remodeling in response to inhaled allergens or nonspecific stimuli. It has significantly increased in prevalence over the past three decades, affecting more than 300 million people globally, and becomes a serious public health problem worldwide [1,2]. Several theories have been proposed to explain the rapid increase of asthma prevalence in developed countries. The most attractive and leading of all explanations is hygiene hypothesis [3]. According to the hygiene hypothesis, unhygienic exposure to microorganisms in childhood may protect from the development of allergic sensitization [4]. Many studies suggest that helminth infection in particular, often elicits immunosuppression of the host, which is presumed to play an important role for the survival of the parasite in the host by regulating immune responses to irrelevant antigens as well as immune protection against concurrent exposed pathogens [5-7]. Therefore, helminth therapy has been tried to ameliorate immunologic diseases including inflammation bowel diseases and reported promising results. Ingestion of *Trichuris suis* eggs has produced a significant improvement of clinical symptoms of Crohn's disease and ulcerative colitis [8,9]. However, there are limitations of helminth therapy, which include the difficulty of preparing eggs or larvae in sterile condition, controversial variable outcomes result from the timing of infection and using asymptomatic dose due to ethical

considerations [10]. The use of parasite-derived products can be an alternative solution to overcome these limitations. According to recent reports, products derived from helminths inhibit the development of allergic responses in mice [5,11]

Caenorhabditis elegans is a free-living, non-parasitic transparent nematode. It is easy to cultivate in large numbers at the laboratory and has short life span of approximately 2-3 weeks. Therefore, *C. elegans* is a useful material in biomedical researches including neuroscience, embryogenesis, and developmental biology. The full genome of *C. elegans* has been completely sequenced, and if products of *C. elegans* were proven to suppress immunologic responses unfavorable to the host, identification of molecular motifs responsible for suppression is highly possible. The previous study reveals that crude extracts of *C. elegans* (CEC) suppress airway inflammation in a murine model of allergic asthma [12]. CEC treatment increased the production of Th1 cytokines such as IL-12 and interferon-γ (IFN-γ), while allergen-induced Th2 cytokine production was decreased in bronchoalveolar lavage fluid (BAL fluid) of mice sensitized with ovalbumin (OVA). The main source of IL-12 is dendritic cells (DCs) and that of IFN-γ is type 1 helper T cells (Th1 cells) in mice treated with both OVA and CEC. Stimulated DCs rapidly begin producing IL-12, which helps naïve CD4+ T helper cells to differentiate towards Th1 cells secreting IFN-γ. Thus, it is speculated that components of *C. elegans* seem to have a suppressive effect on allergic airway inflammation by regulating DCs and T helper

cell differentiation.

Following exposure to other helminths, key changes are observed in the innate immune system of the host, including modification of dendritic cells (DCs) [13,14]. DCs are antigen-presenting cells (APCs) and crucial component of the immune system, which acquire foreign antigen, migrate to the lymph organs, present antigen to T cells and initiate an immune response against pathogens [15]. They induce T cell tolerance, primary T-cell responses and the modulation of T cell-dependent immune responses. Immature DCs are highly endocytic, scan their environment, and express relatively low levels of major histocompatibility complex (MHC) molecules, which present antigens recognized by T cell receptors, and co-stimulatory molecules, such as CD40, CD80, and CD86, required for T cell activation. DCs migrate to lymphoid organs, result in acquiring a mature phenotype characterized by high expression of MHC and co-stimulatory molecules [16].

T cells primed by mature DCs play critical roles in the immune response. Especially, CD4+ T helper cells are major T cell subset which is known to control allergic asthma [17,18]. They give feedback to DCs via co-stimulatory molecules and the secretion of cytokines, promote and maintain responses of CD8+ cytotoxic T cells and support B cells to mount antibody response [19]. Priming and differentiation of T helper cells rely on instructive signals from APC, in particular DCs [20]. Co-stimulatory signals regulate T cell receptor (TCR) - induced CD4+ T cell activation, division and expansion. Cytokines lead T

helper cell differentiation into at least four specific subsets, Th1, Th2, Th17, and regulatory T cells (Treg cells) [21]. IL-12 and IFN-γ direct Th1 differentiation, and IL-4 directs Th2 cell differentiation. IL-1 and IL-6 direct Th17 cell differentiation and IL-10 and TGF-β direct differentiation of Treg cells. Differentiated T helper cell subset is characterized by the basis of the cytokine production profile. Th1 cells secrete IFN-γ and participate in immunity to intracellular bacteria and viruses, whereas Th2 cells produce IL-4, IL-5 and IL-13 and contribute to immunity against helminth infection. Th17 cells secrete IL-17 and are important in antifungal immunity, while Treg cells produce IL-10 and TGF-β in prevention of uncontrolled inflammation and immunopathology in all infections. [21,22]. Of subsets, a number of studies have suggested that the imbalance of Th1/Th2 is responsible for the development and progression of allergic asthma [23,24]. Therefore, one effective therapy for asthma is to promote Th1 immune responses and simultaneously suppress Th2 responses to recover Th1/Th2 balance [25].

In the previous study, CEC showed the suppressive effects on allergic airway inflammation by reducing inflammatory changes in the lung and the production of Th2 type cytokines and IgE. Particularly, CEC treatment enhanced IL-12 and IFN-γ production in vivo [12]. As noted above, DCs are the main resource of IL-12 and deliver the signals to induce Th1 cells producing IFN-γ. Thus, it was assumed that CEC suppress allergic airway inflammation by enhancing Th1 response,

which results from regulating the functions of DCs. However, the details of suppression mechanism of CEC on asthma in mice remain unclear, especially in terms of the possible effect of CEC on interaction between DCs and T helper cells. In this study, therefore, the modulation of activation and cytokine production of DCs by CEC and the ability of DCs to induce T helper cell proliferation and differentiation by CEC treatment were investigated.

MATERIALS AND METHODS

1. Mice

Six to eight-week-old female BALB/c mice were purchased from KOATECH (Seoul, Korea). All mice were maintained under specific pathogen-free condition at Seoul National University College of Medicine, Korea. All animal experiments were approved by the Institutional Animal Care and Use Committee of the Institute of Laboratory Animal Resources, Seoul National University (14-0011-C0A0(4), SNU-150205-1).

2. Culture of bone marrow dendritic cells (BMDCs)

Bone marrow cells were collected from femurs and tibias of mice. Mice were sacrificed by cervical dislocation and femurs and tibias were excised, disinfected with 70% ethyl alcohol for 2 min and washed with cold RPMI 1640 (Welgene, Daegu, Korea). Bone marrow cells were recovered by flushing femurs and tibias with media on ice. Single cell suspension was obtained by vigorous pipetting, filtered with a 70 μm nylon filter and washed with media. Bone marrow cells were depleted of red blood cells by ACK lysis buffer. After final washing, cells were counted and resuspended at 2×10^5 cells/ml in 10 ml complete RPMI1640 media supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco, Grandisland, NY, USA), 1% antibiotics (Gibco),

50 μM 2-mercaptoethanol and 20 ng/ml recombinant mouse Granulocyte macrophage colony-stimulating factor (GM-CSF, Peprotech, Rocky Hill, NJ, USA) and plated in petri dishes. Cells were incubated at 37°C, 5% CO_2 for 7 days. A further 10 ml of 20 ng/ml GM-CSF containing media was added on day 3, and replaced with new media on day 5. On day 7, the non-adherent DCs were harvested, counted for further analysis.

3. Preparation of *C. elegans* crude antigen

The N2 strain of *C. elegans* was grown in nematode growth media (NGM) supplemented with *Escherichia coli* OP50 as a food source. The worms were incubated in an incubator (temperature: 20°C; humidity: 75%). Synchronized adult worms were isolated, washed three times with deionized-distilled water and homogenized in sterile distilled water with a sonicator on ice. Following the centrifugation of the worm homogenate for 20 min at 13,000 rpm in 4°C, the supernatant was incubated with Polymixin B (Thermo Scientific Pierce Protein Research Products, Rockford, IL, USA) for 1 hr in 4°C to eliminate endotoxin contamination. After endotoxin elimination, crude antigen was passed through a 0.45 μm filter for sterilization and kept in –70°C until use. The amount of protein in crude antigen was measured using Nanodrop (Thermo Fisher Scientific Inc., Wilmington, DE, USA). Endotoxin concentration was measured by Pierce LAL chromogenic endotoxin

quantitation kit (Thermo Scientific, Rockford, IL, USA).

4. Antigen stimulation of BMDCs

Immature bone marrow dendritic cells (BMDCs) on day 7 were seeded into 60 mm cell culture plates (Nunc, Rochester, NY, USA) at 1×10^6 cells/ml and stimulated with medium, lipopolysaccharide (LPS, 10 ng/ml), *C. elegans* crude antigen (CEC, 10 μg/ml), or LPS plus CEC for 48 hr at 37°C, 5% CO_2. Cells and supernatants were harvested and assayed for flow cytometric analysis and cytokine production by ELISA, respectively.

5. Separation of CD4+ T cells

T cells were collected from the spleen of syngeneic naïve six to eight-week-old female BALB/c mice. Mice were sacrificed by CO_2 and spleen was excised, ground using 70 μm nylon mesh (Corning Incorporated, Durham, NC, USA), and washed with RPMI1640. Splenocytes were depleted of red blood cells by ACK lysis buffer and the number of cells was counted after washing. T cells were isolated by magnetic selection using the CD4 T cell isolation kit II (Miltenyi Biotec, Auburn, CA, USA). Cells were centrifuged at 300 g for 10 min and supernatant was aspirated. Cell pellet was resuspended in 40 μl of MACS buffer (2 mM EDTA (pH 8.0), 5 mg/ml bovine serum albumin

(BSA, Sigma-Aldrich, Auckland, New Zealand) in PBS) and 10 μl of biotin-antibody-cocktail per 10^7 total cells. After incubation for 5 min in the refrigerator, 30 μl MACS buffer and 20 μl anti-biotin microbeads were added per 10^7 cells. Cells were incubated for 10 min in the refrigerator and cell suspension was applied onto the LS column rinsed previously with 3 ml buffer in the magnetic field of a suitable MACS separator. After washing the column with buffer three times, flow-through containing unlabeled cells was collected, representing the enriched T cells. Cells were centrifuged at 300 g for 10 min and cell pellets were suspended and counted.

CD4+ T cells were isolated by magnetic selection using the CD62L microbeads (Miltenyi Biotec). Cell pellet was resuspended in 90 μl MACS buffer and 10 μl CD62L microbeads per 10^7 cells and incubated for 15 min at 4^oC. Cells were washed by adding 2 ml buffer and centrifuged. Cell pellet was resuspended in 500 μl MACS buffer and was applied on to the column. After removing column from separator, cells were flushed out from column. Cells were centrifuged at 300 g for 10 min and cell pellets were suspended and counted.

6. Co-culture of BMDCs with T cells

Day-7 BMDCs were harvested, seeded at a density of 2×10^4 cells in a 96-well round bottom plate. T cells isolated by magnetic selection were suspended in PBS, added 2 μM CFSE (Carboxyfluorescein

succinimidyl ester; Lifetechnologies, Eugene, Oregon, USA) and mixed by vortexing three times. Cells were incubated at 37°C, 5% CO_2 for 10 min, and 5 ml ice-cold complete media (RPMI1640 media supplemented with 10% FBS and 1% antibiotics) were added. After incubation on ice for 5 min, cells were washed at 1,500 rpm for 5 min twice and the pellet was resusupended with 37°C complete media. Cells were analyzed using a flow cytometer with 488nm excitation and emission filters appropriate for fluorescein.

2×10^5 T cells labeled with CFSE were co-cultured with BMDCs stimulated by media, LPS (10 μg/ml), CEC (10 μg/ml), or LPS and CEC. All media contain anti-CD3e 0.5 μg/ml. Four days later, cells were harvested and assessed for cell proliferation by flow cytometry on a BD FACSCalibur™ (Becton Dickinson). Supernatant of T cells was collected for cytokine production by T cells using ELISA.

7. Flow cytometry analysis

After stimulation with antigens, BMDCs attached to the bottom of the plate were harvested by cell scraper in FACS tubes, washed with PBS containing 0.05% NaN_3 and centrifuged for 4 min in 1,200 rpm. Cells were blocked with unconjugated rat anti-mouse CD16/CD32 (BD2.4G2) for 20 min in the dark at 4°C and then washed with PBS and centrifuged for 4 min in 1,200 rpm. Suspended cells with 400 μl PBS were divided in 4 tubes, each tube containing 100 μl of cells.

Expression of cell surface markers on BMDCs were analyzed by a BD FACSCalibur™ and BD LSRFortessa™ (Becton Dickinson Biosciences) using Pe-Cy5 anti-mouse CD11c (BioLegend N418), APC rat anti-mouse CD11b (BD M1/70), FITC hamster anti-mouse CD80 (BD 16-10A1), PE rat anti-mouse CD86 (BD GL1), PE rat anti-mouse I-A/I-E (BD M5/114.15.2), and FITC rat anti-mouse CD40 (BD 3123). The samples were incubated at 4°C for 30 min in the dark, washed with PBS containing NaN_3.

After co-culture with BMDCs, T cells were harvested, washed with PBS containing 0.05% NaN_3 and centrifuged for 5 min in 1,500 rpm. CFSE-labelled T cells were suspended with 300 μl PBS. The samples were analyzed by a BD FACSCalibur™ and BD LSRFortessa™. Data were analyzed using FlowJo software (TreeStar, Ashland, OR, USA).

8. Cytokine ELISA assay

Sandwich ELISA was used to measure the levels of IL-6, IL-10, IL-12p70, and TNF-α in culture supernatants of BMDCs and the levels of IL-4, IL-5, IL-10, IL-13, IL-17A, TGF-β and IFN-γ in T cell culture supernatants using commercial ELISA kits (eBioscience, San Diego, CA, USA). Each well was coated with 100 μl/well capture antibody overnight at 4°C and blocked with 200 μl 1X diluent for 1 hr at room temperature. After washing with 1X PBST (PBS-Tween20), 100 μl culture supernatant were added to wells and incubated for 2 hr at room

temperature. Detection antibodies, 100 μl, were added to wells after washing and incubated for 1 hr at room temperature. Anti-mouse IgG HRP conjugate, 100 μl, were added to wells after washing and incubated for 30 min at room temperature. 100 ml of TMB were added and the reaction was stopped with 50 μl/well 1M H_3PO_4, after incubation for 15 min. Absorbance was measured at 450 nm using a spectrophotometer (ELISA microplate reader; Molecular devices E max®, Ramsey, MN, USA). Cytokine levels were calculated using standard curves constructed using recombinant murine cytokines. The sensitivity was 4 pg/ml for IL-4, IL-5, IL-6, IL-13 and IL-17A, 8 pg/ml for TNF-α and TGF-β, 16 pg/ml for IL-12 and IFN- γ, and 32 pg/ml for IL-10. All assays were performed in duplicate and the experiment was repeated three times with similar results. The data from the cytokine assay were expressed as an average from three different experiments.

9. Statistical analysis

The data were presented as the means standard deviation (SD). Student's t-test was used to determine the statistical significance of the results obtained. Differences among the comparisons were considered statistically significant when the *P*-value was less than 0.05.

RESULTS

Modulation of BMDC activation by CEC

The immature BMDCs stimulated with LPS showed increased expression of MHCII, CD80, CD86, and CD40. In contrast, BMDCs stimulated with both LPS and CEC showed significantly lower levels of MHCII and co-stimulatory molecules than those stimulated with LPS alone ($P<0.01$). The mean fluorescence intensity (MFI) level of MHCII, CD86, and CD40 was reduced by 44%, 46%, and 38%, respectively, in comparison with that of BMDCs stimulated with LPS alone ($P<0.01$). However, MFI level of CD80 did not show apparent decrease (Figs. 1&2). Furthermore, population of BMDCs exposed to LPS and CEC was divided into $MHCII^{high}$BMDCs and $MHCII^{int}$BMDCs, and $CD86^{high}$BMDCs and $CD86^{int}$BMDCs. BMDCs treated with both LPS and CEC exhibited significantly decreased population of $MHCII^{high}$BMDCs and $CD86^{high}$BMDCs by 52% compared with LPS-treated BMDCs ($P<0.01$) (Fig.3).

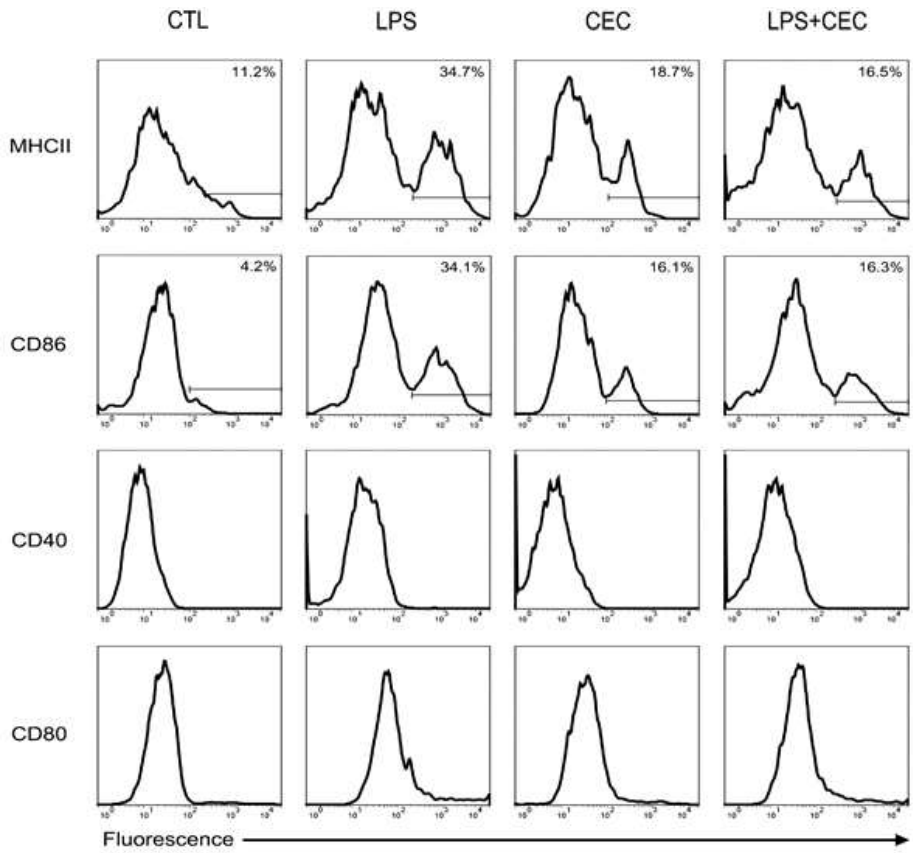

Fig. 1. Expression of co-stimulatory surface markers of bone marrow-derived dendritic cells (BMDCs) in response to stimulation with lipopolysaccharide (LPS) with or without crude extracts of *Caenorhabditis elegans* (CEC). BMDCs were treated with CEC (10 μg/ml) in the presence of LPS (10 ng/ml) for 48 hr. BMDCs were harvested and stained for CD11b, CD11c, MHC class II molecule, and co-stimulatory molecules and analyzed by flow cytometry. The numbers indicate the percentages of $MHCII^{high}$ and $CD86^{high}$ subset among

BMDCs. FACS histograms are representative of three experiments. CTL, control; LPS, BMDCs stimulated with lipopolysaccharide (LPS); CEC, BMDCs stimulated with crude antigen of *Caenorhabditis elegans* (CEC); LPS+CEC, BMDCs stimulated with both LPS and CEC.

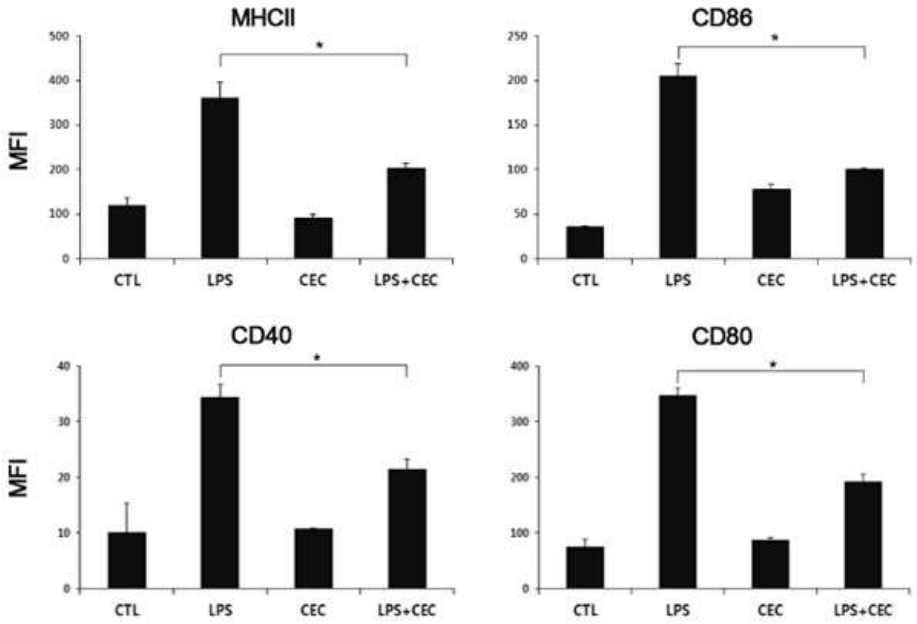

Fig. 2. Mean Fluorescence intensity (MFI) of expression of MHC class II and co-stimulatory molecules by $CD11c^{+}CD11b^{+}$BMDCs. Data shown are means ± SD from three experiments. Statistically significant differences are indicated, **P*<0.01. CTL, control; LPS, BMDCs stimulated with lipopolysaccharide (LPS); CEC, BMDCs stimulated with crude antigen of *Caenorhabditis elegans* (CEC); LPS+CEC, BMDCs stimulated with both LPS and CEC.

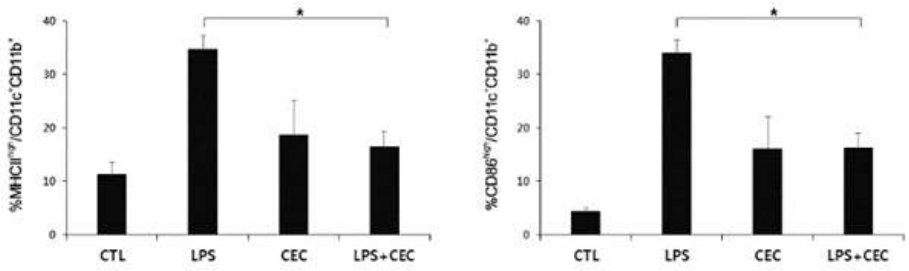

Fig. 3. Frequencies of MHC class II molecule and CD86 expression by CD11c^{+}CD11b^{+}BMDCs. Frequencies of MHC class II molecule and CD86 were analyzed to MHCII^{high}BMDCs and CD86^{high}BMDCs. Data shown are means ± SD from three experiments. Statistically significant differences are indicated, **P*<0.01. CTL, control; LPS, BMDCs stimulated with lipopolysaccharide (LPS); CEC, BMDCs stimulated with crude antigen of *Caenorhabditis elegans* (CEC); LPS+CEC, BMDCs stimulated with both LPS and CEC.

Cytokine production of BMDCs modulated by CEC

Cytokine production by BMDCs with or without CEC treatment was investigated. Stimulation of the immature BMDCs with LPS and CEC showed slightly reduced production of pro-inflammatory cytokines including IL-6 and TNF-α compared to BMDCs with LPS only (P<0.05). However, CEC treatment significantly decreased IL-12p70 production in comparison with LPS treatment alone (P<0.05). Moreover, CEC treatment enhanced production of IL-10, anti-inflammatory cytokines, compared to LPS treatment alone (P<0.05). Notably, IL-10 was secreted at the highest concentration over 1700 pg/ml compared with other cytokines. (Fig. 4)

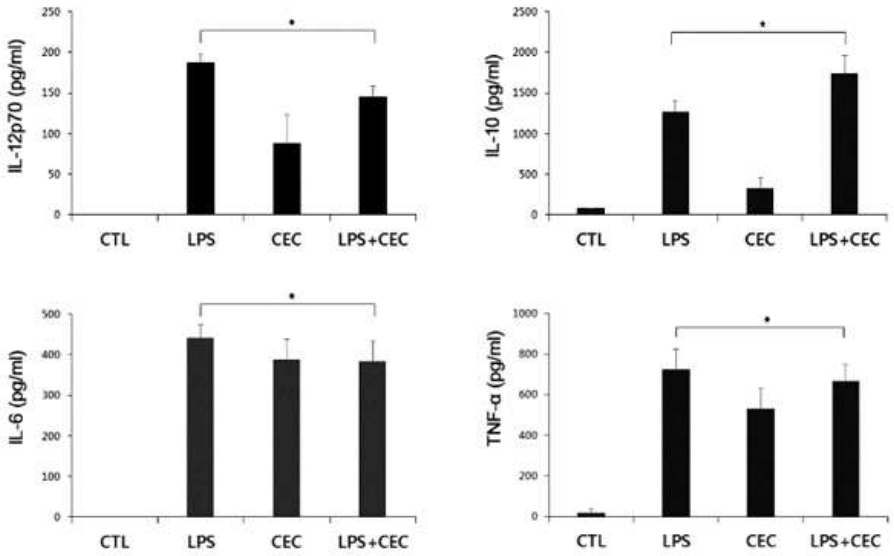

Fig. 4. Production of cytokines by bone-marrow-derived dendritic cells (BMDCs) in response to stimulation with lipopolysaccharide (LPS) with or without crude extracts of *Caenorhabditis elegans* (CEC). Levels of cytokines in cell culture supernatants were measured by ELISA. Data presented are means ± SD from three experiments. Statistically significant differences are indicated, **P*<0.05. CTL, control; LPS, BMDCs stimulated with lipopolysaccharide (LPS); CEC, BMDCs stimulated with crude antigen of *Caenorhabditis elegans* (CEC); LPS+CEC, BMDCs stimulated with both LPS and CEC.

CD4+ T cell proliferation induced by antigen-pulsed BMDCs

To assess the effects of CEC on antigen presenting cell-directed CD4+ T cell proliferation, naïve CD4+ T cells were co-cultured with antigen-treated BMDCs. After four days of co-culture, CD4+ T cells of LPS-pulsed BMDC group showed significant proliferation compared to T cells exposed to BMDCs treated with media. Proliferative response of CD4+ T cells to LPS+CEC-pulsed BMDCs was reduced. Yet, CD4+ T cell proliferation was induced by CEC-pulsed BMDCs (Fig. 5).

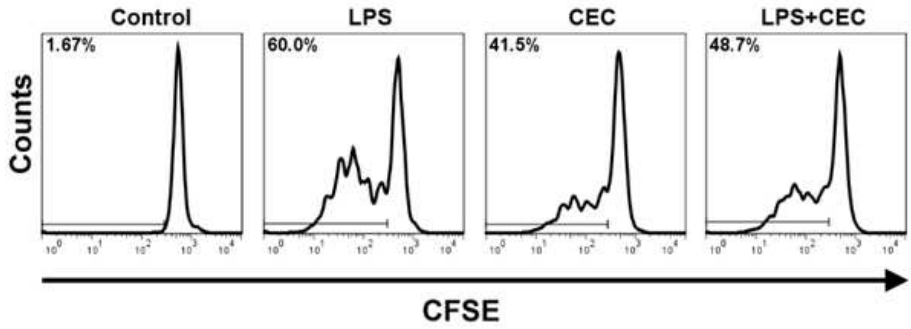

Fig. 5. Proliferation of CD4+ T cells by antigen-pulsed bone marrow-derived dendritic cells (BMDCs). BMDCs were exposed to 10 ng/ml lipopolysaccharide (LPS) and 10 μg/ml *C.elegans* crude antigen (CEC) and co-cultured with CFSE-loaded naïve CD4+ T cells for four days. T cell proliferation was assessed by flow cytometric analysis. Numbers in the histograms indicate the percentages of proliferated T cells. Similar results were obtained in at least four independent experiments. CTL, control; LPS, T cells co-cultured with BMDCs stimulated with lipopolysaccharide (LPS); CEC, T cells co-cultured with BMDCs stimulated with crude antigen of *Caenorhabditis elegans* (CEC); LPS+CEC, T cells co-cultured with BMDCs stimulated with both LPS and CEC.

Cytokine production of CD4+ T cells modulated by CEC

Cytokine production of CD4+ T cells with or without CEC treatment was evaluated. CD4+ T cells with LPS-pulsed BMDCs produced Th1 cytokine (IFN-γ), Th2 cytokines (IL-4, IL-5 and IL-13), Th17 cytokine (IL-17A) and Treg cytokines (IL-10 and TGF-β). Especially LPS is known to bias immune responses towards Th1 differentiation by secreting higher level of IFN-γ. However, T cells stimulated with LPS+CEC-pulsed BMDCs produced significantly higher amounts of IFN-γ than LPS group ($P<0.05$). Moreover, Th2 type cytokines and IL-10 were significantly decreased in LPS+CEC group compared to LPS group ($P<0.05$). However, no difference in production of IL-17A, TGF-β was observed between LPS group and LPS+CEC group. (Fig. 6).

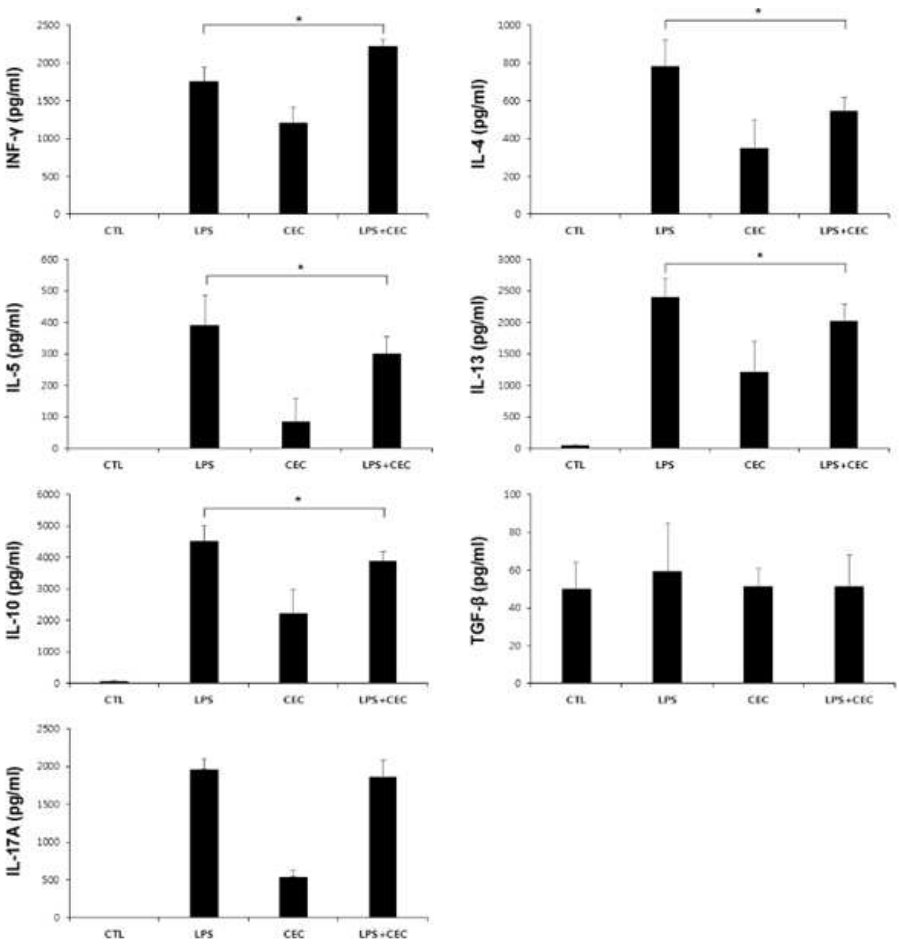

Fig. 6. Cytokine production by CD4+ T cells stimulated by antigen-pulsed bone marrow-derived dendritic cells (BMDCs). BMDCs were exposed to 10 ng/ml lipopolysaccharide (LPS) and 10 μg/ml *C.elegans* crude antigen (CEC) and co-cultured with naïve CD4+ T cells for four days. Levels of cytokines in cell culture supernatants were measured by ELISA. Data presented are means ± SD from three to four experiments. Statistically significant differences are indicated,

*P<0.05. CTL, control; LPS, T cells co-cultured with BMDCs stimulated with lipopolysaccharide (LPS); CEC, T cells co-cultured with BMDCs stimulated with crude antigen of *Caenorhabditis elegans* (CEC); LPS+CEC, T cells co-cultured with BMDCs stimulated with both LPS and CEC.

DISCUSSION

In this study, the effects of CEC was evaluated in terms of function of DCs and an interaction between DCs and CD4+ T cells. BMDCs exposed to LPS with CEC exhibited decreased expression of surface molecules, including MHC class II and co-stimulatory molecules. Pro-inflammatory cytokines production was reduced and anti-inflammatory cytokine, IL-10, was enhanced significantly in the group treated LPS and CEC. Also, CD4+ T cell proliferation was decreased in co-culture with LPS and CEC-stimulated DCs. Interestingly, despite suppression of DC activation, CD4+ T cells co-cultured with both LPS and CEC-stimulated DCs produced significantly higher amount of IFN-γ.

As mention above, expression of MHCII and co-stimulatory molecules on DCs were significantly diminished by administering CEC. Expression of CD80 and CD86 is vital for differentiation of naïve T cells into primed Th2 cells in the process of sensitization in murine asthma model [26]. CD80 and CD86 blockade inhibits CD4+ and CD8+ T cell activation respectively in vivo [27]. Also, CD40 signaling makes DCs more effective antigen presenting cells, by upregulating of MHC class II and co-stimulatory molecules CD80 and CD86 [28]. Given that, reduced expression of surface molecules in both LPS and CEC-stimulated DCs indicates that CEC could interfere with the ability of DCs to prime naïve T cells in drain lymph nodes (dLNs).

Together with expression of surface molecules on DCs, inflammatory cytokines produced by DCs are crucial to interact with T cells. In this study, the level of IL-12p70, IL-6, and TNF-α produced by LPS-stimulated BMDCs were reduced by CEC treatment, suggesting that CEC has ability to downregulate inflammatory response by reducing the secretion of inflammatory cytokines. Similar studies reported that helminths produce products can modulate function of dendritic cells in order to avoid from host protective immunity. Soluble components of *Schistosoma mansoni* and *Trichuris suis* significantly inhibits DC activation and production of IL-6, IL-12 and TNF-α in stimulated human DCs [29,30]. Those of *Trichinella spiralis* results in suppression of DC activation including expression of surface molecules and pro-inflammatory cytokine in mouse DCs [31,32]. Therefore, CEC treatment is suggested to affect T cell response by interfering with the expression of MHCII and co-stimulatory molecules and with production of pro-inflammatory cytokines.

Moreover, CEC significantly elevates production of IL-10 by BMDCs, which is well-known to suppress immune responses for preventing damage to the host [33]. IL-10 inhibits secretion of pro-inflammatory cytokines and upregulation of co-stimulatory molecules by DCs, and suppresses T lymphocyte proliferation in allogeneic mixed lymphocyte reactions [34,35]. Thus, the present results suggest that the inhibitory effect of CEC on DCs is associated with IL-10, which influences T cell responses with surface molecules of DCs.

To determine whether CEC affects these interactions between DCs and T cells, antigen-pulsed DCs were co-cultured with naïve CD4+ T cells. CD4+ T cells cultured with LPS+CEC-pulsed BMDCs showed significantly reduced proliferation compared to those cultured with LPS-pulsed BMDCs. Both the inhibited expression of MHC class II and co-stimulatory molecules on LPS+CEC-exposed BMDCs and reduced T cell proliferation strongly indicates that CEC can modulate immune response by interfering with the function of DCs to induce proliferation of naïve T cells.

Many studies reported that immune suppression by helminth products was regulated by induction of Treg cells, up-regulating TGF-β and $CD4^{+}CD25^{+}Foxp3^{+}$Tcells. Excretory-secretory products of *Trichinella spiralis* suppressed dendritic cell maturation and expanded regulatory T cells in vitro [11], and those of *Fasciola hepatica* induced tolerogenic properties in myeloid dendritic cells and promoted T cell tolerance [36,37]. However, there were no differences in production of TGF-β and lower production of IL-10 in T cells cultured with LPS+CEC-pulsed BMDCs in the present study. Therefore, these observations suggest that Treg cells did not play a role in suppression of the immune response of dendritic cells and CD4+ T cells by CEC treatment.

LPS+CEC-pulsed BMDCs enhanced IFN-γ production, but they showed decreased production of Th2 cytokines in T cells. Therefore,

CEC may influence the interaction between DCs and T cells by modulating the function of DCs which support the development of Th1 cells. Consistent with this speculation, the previous data reported that the IFN-γ level in BAL fluid of mice sensitized with both ovalbumin (OVA) and CEC was significantly higher than in mice sensitized with OVA only. Moreover, the suppressive effect of CEC on asthma induction by OVA was abolished in IFN-γ knock-out mice [12]. This finding suggests that the effect of CEC to suppress allergic response is associated with enhancing Th1 response. IFN-γ is well-known for antiagonism of Th2 differentiation and IgE synthesis, and it restrains allergen-induced eosinophil recruitment into murine lung tissues [38]. Also, it is a downstream mediator of IL-13, hence IL-12-STAT4-IFN-γ axis is important in a Th2 type asthma model [39]. Based on the overview that the alteration of Th1/Th2 ratio is an initial factor for asthma [40], CEC may suppress airway inflammation by restoring the balance of Th1/Th2 cells.

Recent studies showed that several products from helminths or plants attenuated airway inflammation by resetting the Th cell phenotype. ES-62 protects against asthma by up-regulating T-box transcription factor (T-bet) expression and IFN-γ production and inhibiting Th2/Th17 responses [41]. T-bet, a key transcription factor of Th1 cells, promotes Th1 differentiation and IFN-γ production and GATA-binding protein-3 (GATA-3), a key transcription factor of Th2 cells, induces Th2 cell development and production of Th2 cytokines

[42,43]. Concurrently, T-bet negatively regulates GATA-3 and vice versa [44,45]. Some other plant extracts such as mangiferin and ginsenoside Rb1 ameliorated ovalbumin-induced Th2 responses, enhancing Th1 response in vivo. They increased T-bet expression and inhibited GATA-3 [46,47]. Therefore, modulation of T-bet/GATA-3 signaling pathway is important as therapeutic intervention in allergic asthma. Taken together, the protective effect of CEC may be associated with enhancing T-bet signaling to modulate the imbalance of Th1/Th2 cell differentiation.

Meanwhile, because CEC is a complex mixture of antigens, the principal component(s) of CEC must be further investigated. According to recent studies, nematode-derived molecule, cystatin, has been reported to suppress OVA-induced airway inflammation and hyper-reactivity [48]. Cystatin is a cysteine protease inhibitor, which is able to reduce T cell proliferation and regulate production of cytokines. Cystatin from *A. viteae*, ES-62, ameliorates the asthmatic response in an OVA-induced mouse model of asthma, reducing T cell proliferation due to increased secretion of IL-10 [41]. Cystatin of *Oncocerca volvulus* inhibited proliferation of human T cells and that of *Brugia malayi*, Bm-CPI-2, restrained antigen processing of MHCII in human APC [49,50]. Therefore, cystatin may be one of protective molecules in CEC that can suppress antigen presentation of DCs and T cell proliferation.

In conclusion, the present data suggest that CEC can modulate

DCs maturation and production of pro-inflammatory cytokines, resulting in interference with the ability of DCs to prime CD4+ T cells into Th2 differentiation. CEC can interrupt T cell proliferation and lead CD4+ T cells to differentiate into Th1 cells. In this study, CEC showed immunosuppressive effects which is associated with DC-T cell mechanism. Thus, further studies are necessary to identify the molecular motifs of CEC that are responsible for protection against asthma and to clarify the interactions between other immune cells.

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국문초록

연충감염에 의한 숙주의 면역반응조절 및 억제는 널리 보고되었고, 알레르기반응을 억제하는 효과를 보이는 다양한 연충유래물질들이 보고되었다. 이전 연구에서 예쁜꼬마선충 조항원이 천식이 유도된 마우스의 기도염증반응 억제효과를 보임이 보고된 바 있다. 본 연구는 예쁜꼬마선충 조항원이 마우스 수지상세포의 기능과 CD4+ T 세포와의 상호작용에 미치는 효과를 관찰하고자 실시하였다. 예쁜꼬마선충 조항원을 LPS와 함께 마우스 골수에서 유래한 수지상세포에 처리하면 세포막표면 표지단백질인 MHC class II, CD80, CD86, 그리고 CD40의 발현정도가 LPS만을 처리한 양성대조군에 비해 유의하게 감소하였다 (P<0.01). 전염증성 사이토카인인 IL-12p70, IL-6와 TNF-α의 분비가 줄어들었으며 항염증성 사이토카인으로 알려진 IL-10의 분비는 유의하게 증가하였다 (P<0.05). 또한, 예쁜꼬마선충 조항원과 LPS를 함께 처리한 수지상세포로부터 신호를 받은 CD4+ T 세포는 LPS만을 처리한 양성대조군에 비해 덜 증식하였고, Th1 사이토카인인 IFN-γ의 분비를 증가시킨 반면, Th2 사이토카인인 IL-4, IL-5, IL-13의 분비는 더 감소시켰다 (P<0.05). 이상의 결과로 보아 예쁜꼬마선충 조항원은 마우스 수지상세포의 활성화와 사이토카인 분비능을 조절함으로써 CD4+ T 세포의 증식을 감소시키고 활성화된 T세포를 Th1세포로 분화시킨다는 점을 확인할 수 있었다.

주요어: 예쁜꼬마선충 조항원, 수지상세포, CD4+ T cell, 면역 조절

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