



Apple polyphenols suppress antigen presentation of ovalbumin by THP-1-derived dendritic cells

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ABSTRACT

Apple polyphenol extract (AP) and procyanidin contained in AP were investigated for their immunomodulatory effects using THP-1-derived human dendritic cells (TDDCs). The expression levels of HLA-DR (MHC class II) and CD86 (costimulatory molecule) were measured as an indicator of antigen presentation in TDDCs. A significant decrease in HLA-DR expression was observed in the AP and fractionated procyanidin-treated cells in the presence of ovalbumin (OVA), but no effect on CD86 expression was observed. The uptake of OVA was not inhibited by AP treatment, and the gene expression of membrane-associated RING-CH ubiquitin E3 ligase, MARCH1, was up-regulated by AP treatment. It can therefore be presumed that AP suppresses HLA-DR expression via the ubiquitin–proteasome pathway. Furthermore, the up-regulation of IL-12 and TNF- α was found in the procyanidin trimers-treated cells in the presence of OVA. These results suggest that apple polyphenols would be an effective factor for the development of immunomodulatory agents with suppressive effects of antigen presentation.

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1. Introduction

Dendritic cells (DCs) are the most potent antigen-presenting cells (APCs) located in tissues such as skin and mucous membranes and play a critical role in activating T cell-dependent immune responses (Banchereau & Steinman, 1998). DCs are also likely to be essential for the initiation of allergic immune responses. DCs uptake exogenous antigens, process them into immunogenic peptides, and express these peptides for presentation onto major histocompatibility complex (MHC) class II molecules to initiate immune responses. It has also been postulated that DCs are important for the induction of immunologic tolerance (Shortman & Heath, 2001).

Recently, it has been pointed out that dietary factors might contribute to the prevention and treatment of allergic diseases. Typically, the anti-allergic effects of probiotic bacteria, prebiotics, and dietary polyphenols have been demonstrated (Johannsen & Prescott, 2009; Rozy, Jagus, & Chorostowska-Wynimko, 2012; Singh, Holvoet, & Mercenier, 2011). Akiyama et al. (2005) reported that apple polyphenol extract (AP) inhibits the development of food allergies in murine models. AP is mixture of oligomers consisting of chains of flavan-3-ol subunits mainly joined through

C4–C8 (or C6) bonds (Ohnishi-Kameyama, Yanagida, Kanda, & Nagata, 1997). Furthermore, it has been shown that AP had an inhibitory effect on histamine release from rat basophilic leukaemia cells by antigen stimulation (Kanda et al., 1998; Nakano et al., 2008) and that AP intake improved the symptoms of atopic dermatitis in the patients (Kojima et al., 2000). It is presumed that AP has a direct suppressive effect on antigen presentation by APCs such as DCs by inhibiting the cell surface expression of MHC class II and costimulatory molecules, resulting in inefficient antigen presentation, and exerts an anti-allergic effect. However, the direct effect of AP on DCs' function remains unknown.

The current protocol to study the biological function and role of DCs is mainly through differentiation of human donor-derived peripheral blood monocytes with cytokines such as GM-CSF and IL-4 (Casati et al., 2005). Several cell lines including monocytic THP-1 and KG-1 cell lines have been also used as cellular models (Berges et al., 2005). Although a number of studies have been reported for the assessment of the sensitizing potential of chemical allergens using *in vitro*-generated DCs (dos Santos et al., 2009), there are a few studies for the evaluation of allergenicity of food proteins and the screening assay of immunomodulatory agents (Hilmenyuk et al., 2010; Shreffler et al., 2006). The newly developed DCs should be useful tools for quick and easy screening of anti-allergic agents with suppressive effect on antigen presentation. Based on the above theoretical background experimental

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studies of DCs, we successfully obtained DCs from THP-1 cell lines by culturing with PMA and IL-4, and then investigated the effects of AP on antigen presentation of ovalbumin (OVA) and cytokine secretion in THP-1-derived DCs (TDDCs) in this study.

2. Materials and methods

2.1. Materials

AP (procyanidin oligomers, 65.7%; monomeric flavan-3-ols, 12.5%; chalcones, 6.5%; phenolcarboxylic acids, 10.8%) and each procyanidin oligomer fraction (monomers to octamers) (Shoji, Masumoto, Moriichi, Kanda, & Ohtake, 2006) were kindly provided by Asahi Group Holdings (Ibaraki, Japan). Recombinant human interleukin 4 (IL-4) was purchased from PeproTech (Rocky Hill, NJ). Phorbol 12-myristate 13-acetate (PMA) was purchased from Sigma (St. Louis, MO). Other reagents used for flow cytometric analysis were purchased from BD Biosciences (San Jose, CA). All other reagents were of biochemical grade.

2.2. Cell culture and differentiation

Human acute monocytic leukaemia cell line, THP-1, was obtained from JCRB cell bank (Osaka, Japan) and maintained in RPMI 1640 medium supplemented with 10% foetal bovine serum (FBS), 50 U/ml penicillin, and 50 µg/ml streptomycin (Wako, Osaka, Japan) at 37 °C in a humidified atmosphere with 5% CO₂ in air. After culturing from the cell stock, THP-1 cells were used under 10 passages to minimize variability. To induce differentiation into DCs, THP-1 was seeded at 5×10^5 cells/well in a six-well plate and incubated in 2 ml/well in RPMI 1640 medium supplemented with PMA (final concentration, 20 ng/ml) and IL-4 (10 and 20 ng/ml). On day 4, the resulting immature DCs were washed with PBS, stimulated with different concentrations of OVA (0.1, 0.5, and 1 mg/ml) in RPMI 1640 medium supplemented with 10% FBS, 50 U/ml penicillin, and 50 µg/ml streptomycin, and further incubated for 72 h in the absence or presence of AP (10, 50, and 100 µg/ml) and fractionated procyanidins (50 µg/ml).

2.3. Flow cytometry analysis

Expression of cell surface markers was analysed by flow cytometry. Cell staining was performed using the following fluorescence-labelled anti-human monoclonal antibodies (mAbs): FITC-conjugated CD86 (clone BU63) from AbD Serotec (Oxford, UK), Alexa Fluor 647-conjugated HLA-DR (clone L243), CD209 (clone 9E9A8), PerCP/Cy5.5-conjugated CD11c (clone 3.9), and mouse IgG isotype controls from BioLegend (San Diego, CA). Cells were harvested with 1 mM EDTA in PBS, washed with PBS, then incubated with mAbs and appropriated isotypic controls using the manufacturer's recommended dilutions at 4 °C for 30 min after blocking the Fc receptor with FcR blocking reagent (Miltenyi Biotec, Auburn, CA) at 4 °C for 15 min. The stained cells were washed, resuspended with PBS containing 1% BSA, and analysed using FACSCalibur and CellQuest software (BD Bioscience). A total of 10,000 cells were analysed. Expression levels were expressed as delta mean fluorescence intensity (MFI), which was calculated as: MFI of the cells stained with fluorochrome-conjugated antibody – MFI of the background staining cells.

2.4. Analysis of OVA uptake

The immature TDDCs were washed and resuspended in culture medium containing 1 mg/ml FITC-labelled OVA (Sigma) and incubated for 30 min at 37 °C. The cells were washed three times in

cold PBS and then analysed with a FACSCalibur flow cytometer. Uptake of OVA was measured as FITC mean fluorescence intensity (MFI) compared to the control cells. The uptake of FITC-labelled OVA at 4 °C was measured as a negative control.

2.5. Real-time PCR

Total RNA was isolated from the control and AP-treated cells using RNAiso plus Kit (TaKaRa, Japan), and cDNA was generated using the High Capacity RNA-to-cDNA Kit (Applied Biosystems, Carlsbad, CA). cDNA was used as a template for real-time PCR in triplicates with Fast SYBR Green Master Mix and gene-specific primers. PCR and analysis were performed on a StepOne Real-time PCR system (Applied Biosystems). Primer sequences were as follows: β -actin (forward) 5'-CACTATTGGCAACGAGCGGTTC-3', β -actin (reverse) 5'-ACTTGGCGTGCACGATGGAG-3', membrane-associated RING-CH (MARCH) 1 (forward) 5'-TCCCAGGAGCCAGTCAAGGTT-3', MARCH1 (reverse) 5'-CAAAGCGCAGTGTCCTCAGTG-3', MARCH2 (forward) 5'-CTCAGCCTCCAAGTAGCTG-3', MARCH2 (reverse) 5'-CTTGAGGCCAGGACTTTGAG-3', MARCH8 (forward) 5'-ACAGGAAGCCTCCACTTCG-3', MARCH8 (reverse) 5'-GACGTGGAATGTCACTGAG-3'. Cycling was initiated at 95 °C for 1 min, followed by 40 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 1 min. The relative expression of each gene was calculated using the comparative threshold cycle method normalized to β -actin.

2.6. Quantification of cytokine production by ELISA

Culture supernatants were collected after 72 h sample treatment and stored at –20 °C until use. The production of cytokines (IL-12 p70, IL-1 β , TNF- α , and IL-10) was measured by ELISA kit (Bio-Rad Laboratories, Hercules, CA) using 96-well plates according to the manufacturer's instructions.

2.7. Statistical analysis

The results were expressed as the means $\pm$ SD. Statistical evaluation was carried out using an unpaired Student's *t*-test with Statcel software ver. 2.0 (OMS-Publishing, Saitama, Japan).

3. Results

3.1. Generation of immature DCs from THP-1 cells

Human monocytic cell lines, THP-1, were differentiated into immature DCs by culturing with PMA and IL-4 for 4 days. The obtained adherent cells had a typical dendritic morphology (data not shown), and significant expression of CD11c and CD209 (DC-SIGN), surface markers indicative of DCs, was observed in PMA/IL-4 treated cells with the dose dependence of IL-4 (Fig. 1). It was named THP-1-derived DCs (TDDCs) obtained by culturing for 4 days with 20 ng/ml PMA and 20 ng/ml IL-4 and used for further experiments.

3.2. Suppressive effect of AP on antigen presentation by TDDCs

We next investigated whether AP would affect the antigen presentation on TDDCs stimulated with OVA. The expression of MHCII subunit (HLA-DR) and co-stimulatory molecule (CD86) was measured as an indicator of antigen presentation. When TDDCs were stimulated with 1 mg/ml OVA, 1.4- and 1.6-fold increases in the expression of HLA-DR and CD86, respectively, were observed compared with the control cells (Fig. 2). The effect of AP treatment on the expression levels of HLA-DR and CD86 in 1 mg/ml OVA-stimulated TDDCs was then investigated. As shown in Fig. 3, the expression level of HLA-DR was significantly decreased in the TDDCs

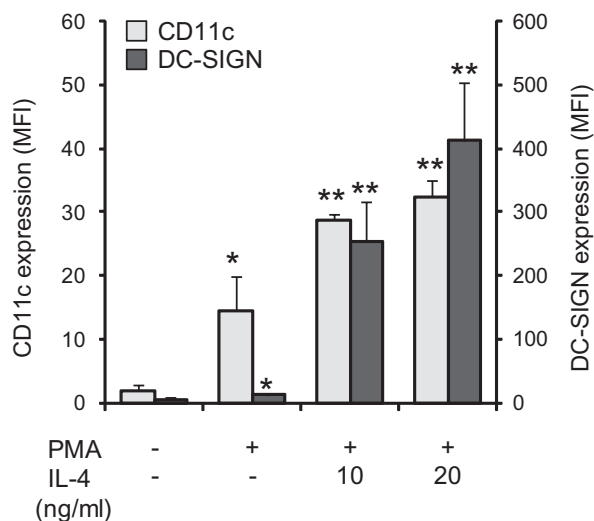


Fig. 1. Changes in surface phenotypes CD11c and DC-SIGN by PMA and IL-4. THP-1 was incubated with PMA (final concentration, 20 ng/ml) and IL-4 (10 and 20 ng/ml). Data are presented as means $\pm$ SD of three independent experiments. * $p < 0.05$ and ** $p < 0.01$, when compared with the control cells.

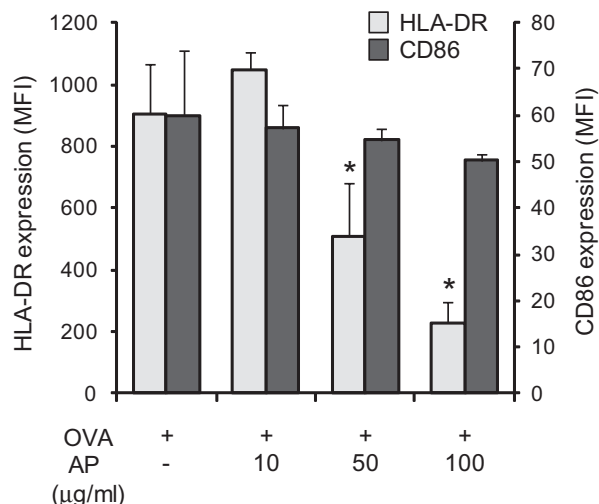


Fig. 3. Suppressive effects of apple polyphenol extract (AP) on the expression of HLA-DR and CD86 in OVA-stimulated THP-1-derived human dendritic cells (TDDCs). TDDCs were treated with AP (10, 50, and 100 µg/ml) in the presence of OVA for 72 h. Data are presented as means $\pm$ SD of three independent experiments. * $p < 0.05$, when compared with the control cells.

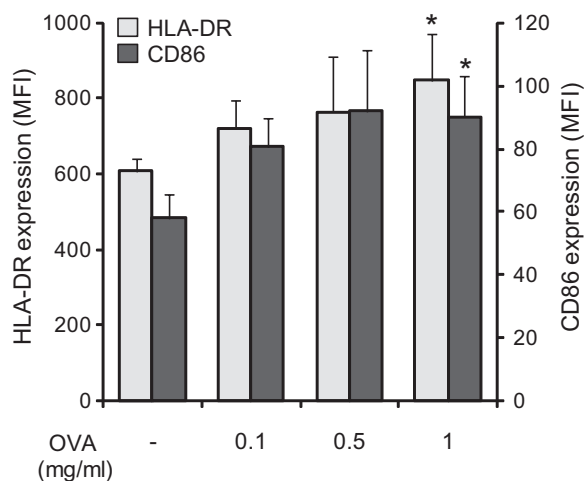


Fig. 2. Effect of ovalbumin (OVA) on the expression of surface phenotypes HLA-DR and CD86. THP-1-derived human dendritic cells (TDDCs) were stimulated with OVA (0.1, 0.5, and 1 mg/ml) for 72 h. Data are presented as means $\pm$ SD of three independent experiments. * $p < 0.05$, when compared with the control cells.

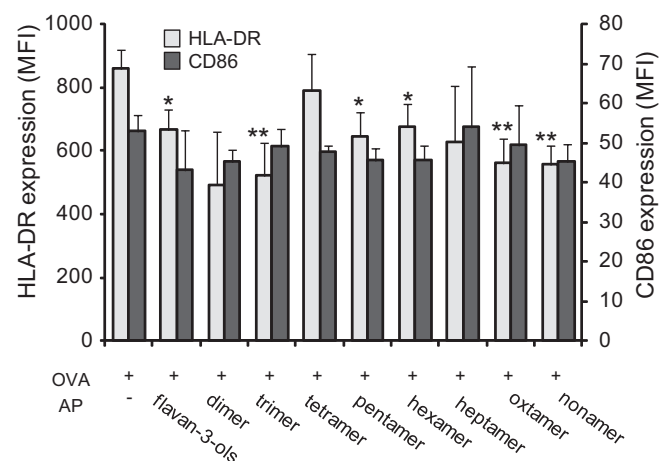


Fig. 4. Suppressive effects of the fractionated procyanidins on expression of HLA-DR and CD86 in OVA-stimulated TDDCs. TDDCs were treated with fractionated procyanidins (50 µg/ml) in the presence of OVA for 72 h. Data are presented as means $\pm$ SD of two independent experiments. * $p < 0.05$ and ** $p < 0.01$, when compared with the control cells.

treated with 50 and 100 µg/ml AP in the presence of OVA. On the other hand, the expression level of CD86 slightly decreased, but the difference was not statistically significant.

We further investigated the relationship between the polymerization degree of procyanidins in AP and the suppressive effect of antigen presentation on TDDCs stimulated with OVA. Individual fractions consisting of monomeric flavan-3-ols ((+)-catechin and (-)-epicatechin), dimers to nonamers were employed for the measurement (Fig. 4). All fractions (50 µg/ml) suppressed the expression of HLA-DR in OVA-stimulated TDDCs, and especially trimer, octamer, and nonamer procyanidins exhibited higher suppressive effects ($p < 0.01$), compared with the other oligomer fractions. However, no clear relationship between the polymerization degree and the suppressive effects on HLA-DR expression was observed. In contrast, all fractions showed no significant decrease in CD86 expression.

3.3. Effect of AP on OVA uptake of TDDCs

To determine whether AP inhibits the uptake of OVA by TDDCs, the changes of MFI during FITC-labelled OVA in the presence and absence of AP were investigated. When cells were incubated in the medium containing FITC-labelled OVA at 4 °C as a control, the MFI was 7.55 ± 0.55 , indicating that OVA uptake was not observed (data not shown). OVA was efficiently taken up by TDDCs compared with the control cells, however, the increased MFI of FITC-OVA in TDDCs remained unchanged after treatment with AP. These results suggest that the OVA uptake by TDDCs was not affected by treatment with AP.

3.4. Effect of AP on ubiquitin gene expression

MARCH proteins are E3 ubiquitin ligases that have been reported to down-regulate several surface molecules, including

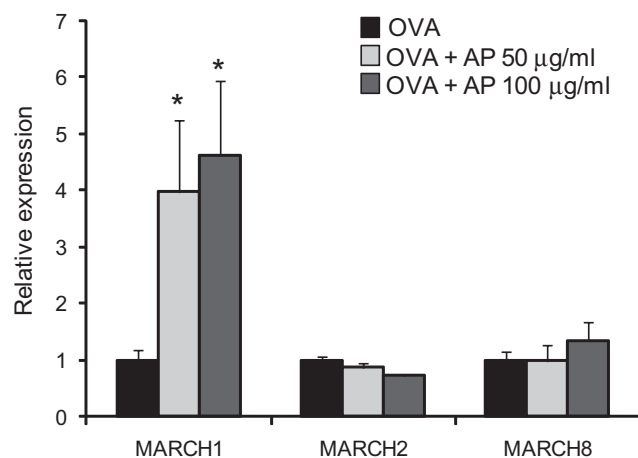


Fig. 5. Effect of AP on membrane-associated RING-CH (MARCH) family gene expression of OVA-stimulated TDDCs. TDDCs were treated with AP (50 and 100 µg/ml) in the presence of OVA for 72 h. The gene expression levels of MARCH1, 2, and 8 were quantified by real-time PCR. Data are presented as means $\pm$ SD of two independent experiments. * p < 0.05, when compared with the control cells.

MHC class I and II, and CD86 (Ohmura-Hoshino et al., 2006). We hypothesized that the marked decrease in HLA-DR expression by AP would be caused by activation of the ubiquitin–proteasome pathway. As shown in Fig. 5, a significant up-regulation of MARCH1 gene expression was observed in the AP-treated cells; however, MARCH2 and MARCH8 mRNA levels showed no significant changes. This result suggests that an increase in MARCH1 mRNA in TDDCs resulted in a marked decrease of HLA-DR expression.

3.5. Cytokine secretion profiles of AP-treated TDDCs

We further examined whether AP and their fractionated procyanidins affected the cytokine secretion from TDDCs in the presence of OVA. Treatment with AP led to a significant increase of IL-12 and TNF- α secretion (Fig. 6A and B), whereas a significant decrease in IL-10 secretion was observed in AP-treated cells (Fig. 6B). In addition, there was no significant change in the IL-1 β secretion level among the AP and oligomer-treated cells (Fig. 6A). Particularly, sig-

nificant up-regulations of IL-12 and TNF- α and down-regulation of IL-10 were found in the procyanidin trimers-treated cells in the presence of OVA.

4. Discussion

The initiation of antigen-specific T cell responses requires stimulation of the T cell receptor via its peptide/MHC class II molecule and the delivery of co-stimulatory signals (Allison, 1994). The present study demonstrated *in vitro* that apple procyanidins could suppress the expression of HLA-DR, the predominant isotype of MHC class II molecule, in OVA-stimulated human DCs; however, they had no effect on the expression of CD86, the major molecule with co-stimulatory activity (Zhang, Martin, Yang, Michalek, & Katz, 2004).

The MARCH genes were considered to be important in the down-regulation of membrane receptors such as MHC class II, CD86 and ICAM 1 (Nathan & Lechner, 2009). The MARCH family consists of 11 genes, and MARCH1 was identified as key facilitators in MHC class II regulation, as exogenous expression of MARCH1 ubiquitylates cell surface MHC class II, initiating its internalization and lysosomal degradation (Ohmura-Hoshino et al., 2006). The present data clearly showed that the up-regulation of MARCH1 occurs by spiked AP on TDDCs. It is therefore reasonable to suggest that AP could degrade the MHC molecules through ubiquitylation. Additionally, the expression of MARCH2 and MARCH8 in OVA-stimulated TDDCs was not observed in the addition of AP. Since expression of MARCH2 and MARCH8 was associated with CD86 degradation (Ohmura-Hoshino et al., 2006), this finding also supports that AP would not affect the expression of CD86 onto TDDCs.

TNF- α and IL-1 β are known to be a proinflammatory cytokine (Miller, Maletic, & Raison, 2009; Sims & Smith, 2010). In this study, we showed that AP had no effect on IL-1 β secretion in TDDCs in the presence of OVA, although AP up-regulated the TNF- α secretion. Boisleve, Kerdine-Romer, and Pallardy (2005) showed that TNF- α plays important roles in the maturation of human DCs, including up-regulation of co-stimulatory molecule (CD86) and maturation marker (CD83), as well as the chemokine receptor CCR7, via activation of p38 MAPK signalling pathway. Our studies indicated that AP had no effect on the CD86 expression in OVA-stimulated

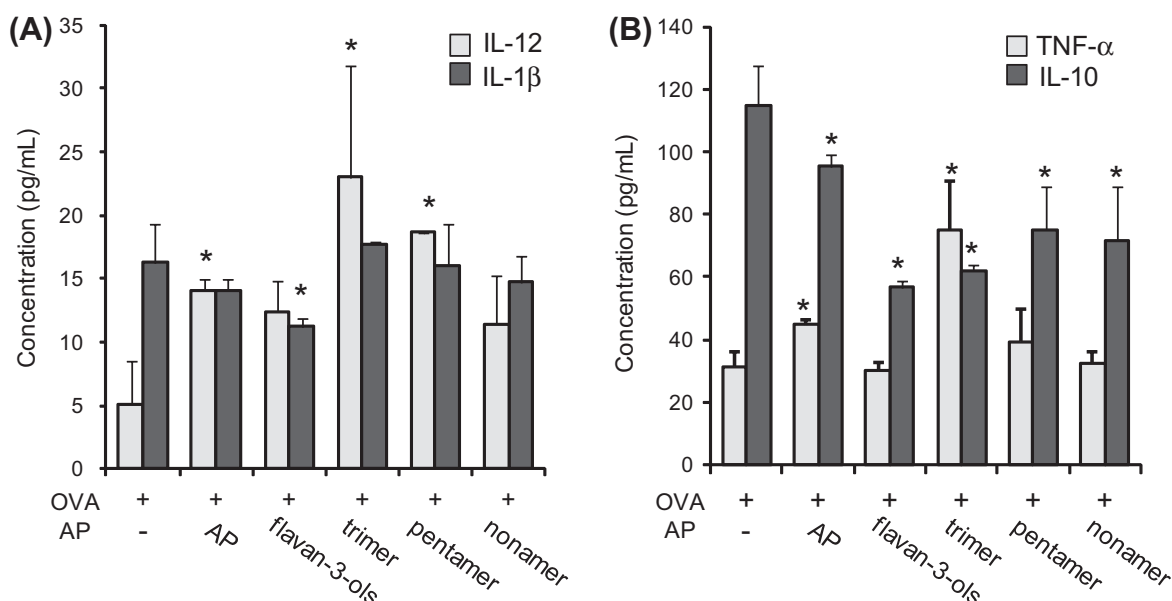


Fig. 6. Changes in secretion profiles of IL-12 and IL-1 β (A), TNF- α and IL-10 (B) in OVA-stimulated TDDCs by AP treatment. TDDCs were treated with AP and the fractionated procyanidins (50 µg/ml) in the presence of OVA for 72 h. The culture supernatant was measured for IL-12, IL-1 β , TNF- α , and IL-10 secretion by ELISA. Data are presented as means $\pm$ SD of two independent experiments. * p < 0.05, when compared with the control cells.

TDDCs. Taken together, it is suggested that increased TNF- α secretion might be correlated with a constant level of CD86 expression in AP-treated TDDCs in the presence of OVA.

In this study, the mechanistic link to T cell presentation does not clear, and it is an important issue to be considered for further experiments. Native T helper (Th) cells are traditionally thought to differentiate into effector T cell subsets, such as Th1, Th2, Th17, and Treg cells, after maturational process induced by antigenic stimulation (Zhou, Chong, & Littman, 2009). It depends on complex interactions with APCs and cytokine signalling. The present results indicated that secretion level of IL-12, a key inducer of differentiation towards the Th1 phenotype (Zhou et al., 2009), was up-regulated by the treatment with AP, thereby inhibiting Th2 differentiation. IL-10 is known as one of the Th2 cytokines (Paul & Zhu, 2010), and the down-regulation of IL-10 was found in AP-treated cells. Therefore, we speculate that AP could be involved in the regulation of Th1/Th2 differentiation.

Regulatory T (Treg) cells are known to be critical regulators of immune tolerance (Sakaguchi, Yamaguchi, Nomura, & Ono, 2008). Treg cells are able to inhibit the development of allergic Th2 response. IL-10 is produced by both T cells and APCs, and IL-10 is required for Treg cells to mediate tolerance (Kushwah & Hu, 2011). Human DCs express aldehyde dehydrogenase (ALDH) that can induce native T cells to Foxp3⁺ Tregs; however, no change in the expression level of ALDH by AP treatment was observed (data not shown). Therefore, these results suggest AP might not affect induction of immune tolerance.

Nakano et al. (2008) have reported that AP suppresses mast cell degranulation by inhibiting IgE-Fc ϵ RI binding and that procyanidin trimers exhibit the strongest inhibitory effects. Our results also indicated the significant suppressive effects of procyanidin trimers on antigen presentation on TDDCs stimulated with OVA. After oral administration, procyanidins were absorbed and detected in the plasma of humans and rodents (Sano et al., 2003; Shoji, Masumoto, Moriichi, Akiyama, et al., 2006). The gut absorption of procyanidins depends on their degree of polymerization, and the procyanidin dimers and trimers was easily absorbed in the small intestine, compared with that of procyanidin polymers, with an average polymerization degree of 6 (Deprez, Mila, Huneau, Tome, & Scalbert, 2001). Therefore, our findings suggest that oral administration of AP contained procyanidin trimers that would be an effective factor for the treatment or prevention of allergic disease.

In conclusion, we demonstrated *in vitro* that apple-derived procyanidins could suppress the antigen presentation of OVA by human DCs. It is caused by degradation of MHC class II via the ubiquitin–proteasome pathway. This study suggests that the procyanidins-enriched apple extract would be an effective factor to prevent and treat allergic diseases.

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