Table 3. Primers and Probes Used in This Study

Targeted species	Name[a]	Sequence of oligonucleotide (5′-3′)[b]	Amplicon length
O. guepiniformis	OMGU-F	5′-TCTGGGCTTCTATGTCTTACAAACTC-3′	101 bp
	OMGU-R	5′-CCGTTGCTGAAAGTTGTATAAGTTTT-3′	
	OMGU-Taq	5′-(FAM)-CTCTTTATTGGTACTTAATTG-(NFQ)-(MGB)-3′	
L. edodes	LEED-F	5′-CATCCACCTGTGCACTTTTTGTAG-3′	93 bp
	LEED-R	5′-GAAGCCTTGTCAACTAGTCTTTTCAA-3′	
	LEED-Taq	5′-(VIC)-AGGTGCTCATTATGAGTTA-(NFQ)-(MGB)-3′	

[a]F: Forward primer, R: Reverse primer, Taq: TaqMan MGB probe
[b]FAM: 6-carboxyfluorescein, VIC: 6-carboxyrhodamine, NFQ: non-fluorescent quencher, MGB: minor groove binder

primer pairs and TaqMan MGB probes. The concentrations of both primer pairs were tested in the range of 100–400 nM (100, 200, 300 and 400 nM) and no other parameters were varied, including the concentrations of both probes (200 nM). Almost no difference was apparent in both the *O. guepiniformis* and *L. edodes* detection systems between the Ct values at primer concentrations of 300 and 400 nM, although these values were lower at 300 and 400 nM than those at 100 and 200 nM. The optimal concentration of both primer pairs was therefore determined to be 300 nM.

Setting the concentration of both primer pairs at 300 nM, the concentrations of both probes were then tested in the range of 50–200 nM (50, 100 and 200 nM). Almost no difference was apparent between the Ct values obtained at probe concentrations of 100 and 200 nM with both detection systems, although the Ct values were lower at 100 and 200 nM than at 50 nM. The optimal reaction conditions were therefore determined to be 300 nM for the primer pairs and 100 nM for the probes.

Specificity

The designed primer pairs and TaqMan MGB probes were tested for their specificity toward the target species and for their cross-reactivity with other species of mushroom and with common food items. Multiplex real-time PCR was performed by using DNA extracted from the fruiting bodies of 17 *O. guepiniformis*, 16 *L. edodes* and 57 other species (Tables 1 and 2). The *O. guepiniformis* samples gave Ct values in the range of 17.50–20.28, and the *L. edodes* samples gave Ct values in the range of 16.51–19.21 (data not shown). Ct values were obtained from all the samples of *O. guepiniformis* and *L. edodes*, and no cross-reaction was apparent for any non-target species in either detection system. These results suggest that the designed primer pairs and probes were highly specific and selective for their target species and exhibited no cross-reactivity with other species.

Sensitivity and linearity

The limit of detection (LOD) and linearity of the standard curves for both the *O. guepiniformis* and *L. edodes* detection systems were determined by using a 10-fold dilution series of *O. guepiniformis* and *L. edodes* DNA ranging from 2.5 to 0.00025 ng. LOD was 0.00025 ng of DNA for both systems. Ct values obtained from the 10-fold dilution series were plotted against the logarithm of the absolute amount of DNA (ng) to obtain the standard curves. The linearity of both detection systems ranged from 2.5 ng to 0.00025 ng of DNA with a square regression correlation (R^2) of >0.999 (Fig. 1). PCR efficiency of 100% is achieved when the slope is close to the theoretical value of −3.32. The observed slopes (−3.58 for *O. guepiniformis* and −3.58 for *L. edodes*) shown in Fig. 1 are close to this theoretical value. Based on this data, a PCR efficiency of 90% for the *O. guepiniformis* and *L. edodes* detection systems was calculated by using the equation PCR efficiency $= (10^{-1/\text{slope}} - 1) \times 100$. In order to mimic the conditions present during the extraction of *O. guepiniformis* and *L. edodes* DNA from different foods, genomic DNA of each species was mixed with genomic DNA of *Panellus serotinus* (Mukitake) at five mixing levels (0.01, 0.1, 1, 10 and 100%). The amount of total template DNA (*Panellus serotinus* DNA plus *O. guepiniformis* or *L. edodes* DNA (0.00025, 0.0025, 0.025, 0.25 or 2.5 ng)) was adjusted to 2.5 ng. The LOD value was 0.01% (0.00025 ng of DNA of *O. guepiniformis* or *L. edodes*), and the linearity of both detection systems was in the range of 0.01–100% with a square regression correlation (R^2) of >0.999 (data not shown). The PCR efficiency of the *O. guepiniformis* and *L. edodes* detection systems was greater than 80%, as calculated by the foregoing equation (a slope of −3.85 for *O. guepiniformis* and −3.82 for *L. edodes*). These results suggest that both detection systems were highly sensitive and gave good linearity for the standard curves, as well as high PCR efficiency for detecting *O. guepiniformis* and *L. edodes*.

Application of the assay to samples of processed foods containing other food items and artificial gastric juice

The applicability of the assay to processed mushrooms was investigated by analyzing samples of raw and processed (baked, stir-fried, deep-fried, boiled, and digested) fruiting bodies of *O. guepiniformis* and *L. edodes*. DNA extracted from these samples was tested by using the multiplex real-time PCR assay described in this current study. The Ct values obtained for the processed samples ranged from 20.48 to 26.55 (18.55 for the raw *O. guepiniformis* sample) with the *O. guepiniformis* detection system and from 17.79 to 22.73 (17.16 for the raw *L. edodes* sample) with the *L. edodes* detection system (Table 4). Although all processed samples of *O. guepiniformis* and *L. edodes* had higher Ct values than the corresponding raw samples, Ct values with sufficient sensitivity for detection were still obtained. The applicability of the assay for processed mushrooms containing other food items and gastric juice was investigated by using miso soup samples containing vegetables with approximately 1% (w/w) *O. guepini-*

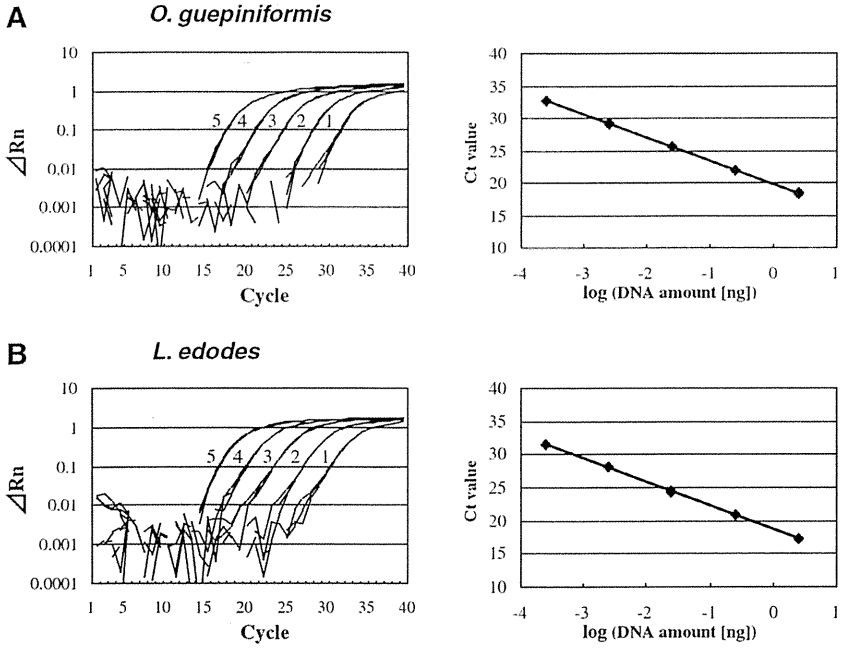

Fig. 1. Amplification Plots and Standard Curves for Multiplex Real-Time PCR.
The amplification plots were generated by 10-fold serial DNA dilutions of *O. guepiniformis* (A) and *L. edodes* (B) DNA (1, 0.00025 ng; 2, 0.0025 ng; 3, 0.025 ng; 4, 0.25 ng; 5, 2.5 ng). The standard curves were generated from the amplification data. The relationships between threshold cycle (Ct) and log DNA amount are $y = -3.58x + 19.83$ ($R^2 = 1.000$) (A) and $y = -3.58x + 18.68$ ($R^2 = 1.000$) (B).

Table 4. Ct Values of Processed Mushrooms by Multiplex Real-Time PCR

Processing method	*O. guepiniformis* Mean Ct value ± SD[a]	*L. edodes* Mean Ct value ± SD[a]
None	18.55 ± 0.03	17.16 ± 0.01
Baking at 240 °C for 2 min on hot plate	20.48 ± 0.02	18.34 ± 0.04
Stir-frying at 240 °C for 2 min on hot plate	26.55 ± 0.02	22.73 ± 0.06
Deep-frying at 180 °C for 2 min in oil	21.14 ± 0.02	21.53 ± 0.04
Boiling for 30 min in water	21.71 ± 0.05	18.94 ± 0.04
Boiling for 60 min in water	21.16 ± 0.02	17.79 ± 0.02
Boiling for 120 min in water	24.92 ± 0.03	17.79 ± 0.02
Boiling for 180 min in water	23.08 ± 0.05	17.90 ± 0.04
Digesting raw mushroom	19.00 ± 0.02	17.99 ± 0.01
Miso soup spiked with 1% mushroom[b]	25.71 ± 0.09	25.18 ± 0.02
Digested miso soup spiked with 1% mushroom[b]	26.60 ± 0.07	25.93 ± 0.07

[a]Mean Ct value and SD of triplicate wells for each sample
[b]*O. guepiniformis* or *L. edodes*

formis or *L. edodes* and artificially digested samples of *O. guepiniformis* and *L. edodes*. The Ct values obtained from these samples ranged from 25.18 to 26.60 (Table 4) with the *O. guepiniformis* and *L. edodes* detection systems. These results indicate that the multiplex real-time PCR assay described in this current study was applicable for use in the identification of processed and digested fruiting bodies of *O. guepiniformis* and *L. edodes*, and of fruiting bodies of *O. guepiniformis* and *L. edodes* in processed foods containing other food items and gastric juice.

Repeatability and reproducibility of multiplex real-time PCR

The repeatability and reproducibility were evaluated by using a 10-fold dilution series of both *O. guepiniformis* and *L. edodes* DNA ranging from 2.5 to 0.00025 ng. The mean, standard deviation (SD), and coefficient of variation (CV) of the Ct values for repeatability were calculated from the mean values obtained from three replicates performed by one researcher on the same day. The same variables for the Ct values of reproducibility were calculated from the mean values obtained from replicates performed over three separate days of trials. In respect of the repeatability, CV of the Ct values for *O. guepiniformis* DNA ranged from 0.04 to 0.38%, whereas CV of the Ct values for *L. edodes* DNA ranged from 0.05 to 0.30%. In respect of the reproducibility, CV of the Ct values for *O. guepiniformis* DNA ranged from 0.20 to 1.23%, whereas CV of the Ct values for *L. edodes* DNA ranged from 0.21 to 0.85% (Table 5). These results for the repeatability and reproducibility tests indicate that the multiplex real-time PCR assay was reliable in its detection of *O. guepiniformis* and *L. edodes* DNA.

Table 5. Repeatability and Reproducibility of Multiplex Real-Time PCR

DNA dilution (ng)		*O. guepiniformis*				*L. edodes*			
		Same day ($n = 3$)			3 days ($n = 9$)	Same day ($n = 3$)			3 days ($n = 9$)
		1	2	3		1	2	3	
2.5	mean Ct	18.40	18.45	18.38	18.41	17.24	17.24	17.19	17.22
	SD	0.02	0.01	0.04	0.04	0.02	0.02	0.05	0.04
	CV	0.08	0.05	0.24	0.20	0.13	0.13	0.28	0.21
0.25	mean Ct	21.97	22.24	22.13	22.11	20.84	21.21	21.18	21.08
	SD	0.01	0.01	0.05	0.12	0.06	0.02	0.03	0.18
	CV	0.05	0.05	0.21	0.54	0.30	0.11	0.13	0.85
0.025	mean Ct	25.55	26.03	26.27	25.95	24.39	24.58	24.57	24.51
	SD	0.02	0.03	0.03	0.32	0.02	0.07	0.05	0.10
	CV	0.08	0.10	0.10	1.23	0.09	0.29	0.21	0.43
0.0025	mean Ct	29.21	29.54	29.97	29.57	28.08	28.27	28.32	28.23
	SD	0.06	0.10	0.01	0.34	0.02	0.02	0.01	0.11
	CV	0.21	0.35	0.04	1.13	0.06	0.08	0.05	0.40
0.00025	mean Ct	32.67	33.09	33.42	33.06	31.54	31.85	31.93	31.77
	SD	0.12	0.05	0.13	0.34	0.03	0.09	0.03	0.19
	CV	0.35	0.16	0.38	1.02	0.09	0.27	0.09	0.58

Discussion

Wild fruiting bodies of the poisonous mushroom *O. guepiniformis* are frequently misidentified as those of such an edible mushroom as *L. edodes*, resulting in cases of food poisoning throughout Japan. In cases of suspected mushroom poisoning, species identification is commonly achieved through an examination of the morphological characteristics; however, this is often impossible when mushroom samples have been processed, or when only residual amounts of the food items and vomit remain. If a morphological examination is inconclusive, an assay without depending on morphology may be required to verify the cause of mushroom poisoning. Real-time PCR assays have proved useful for identifying mushroom species independently of morphology.[8,12,27] A multiplex real-time PCR assay using DNA probes has been able to distinguish such different species as *O. guepiniformis* and *L. edodes* in one run, allowing for rapid detection, reduced costs and less sample waste when compared to similar assays using simplex PCR.

O. guepiniformis has been identified in the past with a real-time PCR assay using SYBR Green I;[8] however, the detection of highly homologous target sequences such as those in the ITS regions of *Omphalotus* DNA by using SYBR Green I is difficult, and this method is not applicable to multiplex real-time PCR. Moreover, a real-time PCR assay using SYBR Green I has not yet been applied to clinical samples such as those that have been exposed to gastric juices, or to processed food samples containing other food items. Identifying *O. guepiniformis* in samples such as these may be required in order to determine the cause of food poisoning.

A multiplex real-time PCR assay using TaqMan MGB probes was developed in the present study for simultaneously detecting *O. guepiniformis* and *L. edodes* with high specificity. The detection of target sequences using TaqMan MGB probes is more specific than detection using SYBR Green and standard DNA probes.[9–11] No cross-reaction was apparent with any non-target species listed in Tables 1 or 2 in a cross-reactivity test. Among the 57 species listed in Table 2, 52 are frequently used in cooking; the other five species (*Amanita pantherina*, *Clitocybe acromelalga*, *Entoloma rhodopolium*, *Omphalotus olearius* and *Tricholoma ustale*) are poisonous and are often the cause of mushroom poisoning in Japan.[4] In addition, all the target species used for the test (OG1-17 and LE1-16) were detected by the assay. Although some species of genus *Omphalotus* were not available, the sequences of the TaqMan MGB probes were confirmed to have no significant homology with the known sequences of other species of genus *Omphalotus* as determined by a BLAST search.

The assay described in the current study proved effective for detecting *O. guepiniformis* and *L. edodes* in processed and digested samples, as well as in processed food samples containing other food items and artificial gastric juice. Ct values were obtained from all the processed and digested fruiting bodies, as well as from miso soup samples containing 1% fruiting bodies and artificially digested samples (Table 4). The Ct values obtained from all the processed and digested fruiting bodies were higher than those obtained from the raw fruiting bodies, suggesting the presence of some genomic DNA degradation in the processed and digested samples caused by high temperature and the presence of acid in the artificial gastric juice.[29–33]

This assay proved to have high sensitivity and good linearity at low DNA concentrations (0.00025–2.5 ng) (Fig. 1) due to the ITS region chosen as the target sequence possessing multiple copies.[25] The assay also provided sufficient accuracy with good repeatability (CV of Ct values = 0.04–0.38%) and reproducibility (CV of Ct values = 0.20–1.23%) (Table 5).

The proposed method can detect 0.01% *O. guepiniformis* DNA in the mixture of genomic DNAs extracted from *Panellus serotinus* and *O. guepiniformis* DNA, as well as 0.01% *L. edodes* DNA in the mixture of genomic DNAs extracted from *Panellus serotinus* and *L. edodes* DNA. The method can also detect 1% *O. guepiniformis* DNA and 1% *L. edodes* DNA in the mixture of *O. guepiniformis* DNA and *L. edodes* DNA (data not shown). This method would therefore be applicable to a processed sample involving food poisoning which would contain more than 1% *O. guepiniformis* in a practical case of accidental poisoning.

In conclusion, the current study presents a rapid and target-specific multiplex real-time PCR assay able to

simultaneously detect the presence of *O. guepiniformis* and *L. edodes*. This novel PCR assay has high specificity for the target species, high sensitivity, good linearity and applicability to processed samples. These characteristics would make the assay useful for rapidly identifying *O. guepiniformis* and *L. edodes*.

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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.

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% CO_2 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′-ACTTGCGGTGCACGATGGAG-3′, membrane-associated RING-CH (MARCH) 1 (forward) 5′-TCCCAGGAGCCAGTCAAGGTT-3′, MARCH1 (reverse) 5′-CAAAGCGCAGTGTCCCAGTG-3′, MARCH2 (forward) 5′-CTCAGCCTCCCAAGTAGCTG-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-1β, 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 ± 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

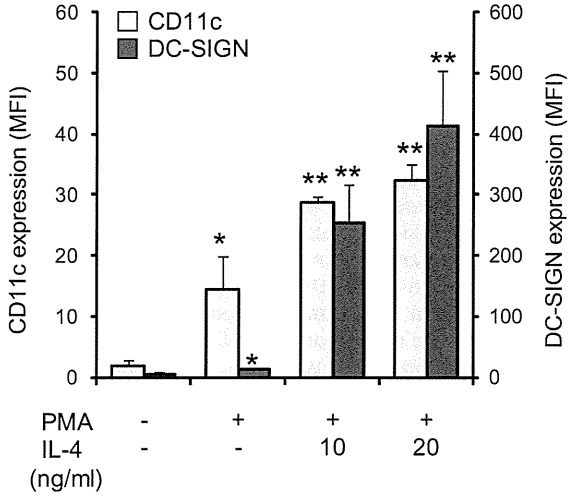

Fig. 1. Changes in surface phenotypes CD11c and DC-SIGN by PMA and IL-4. THP-1 was incubated with PMA (final concentration, 20 ng/ml) and IL-4 (10 and 20 ng/ml). Data are presented as means ± SD of three independent experiments. $^{*}p < 0.05$ and $^{**}p < 0.01$, when compared with the control cells.

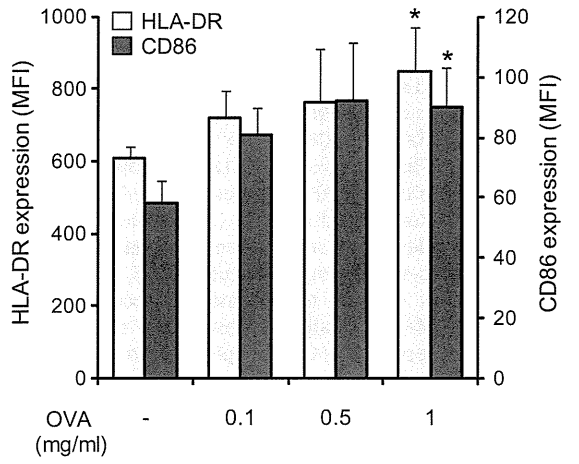

Fig. 2. Effect of ovalbumin (OVA) on the expression of surface phenotypes HLA-DR and CD86. THP-1-derived human dendritic cells (TDDCs) were stimulated with OVA (0.1, 0.5, and 1 mg/ml) for 72 h. Data are presented as means ± SD of three independent experiments. $^{*}p < 0.05$, when compared with the control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

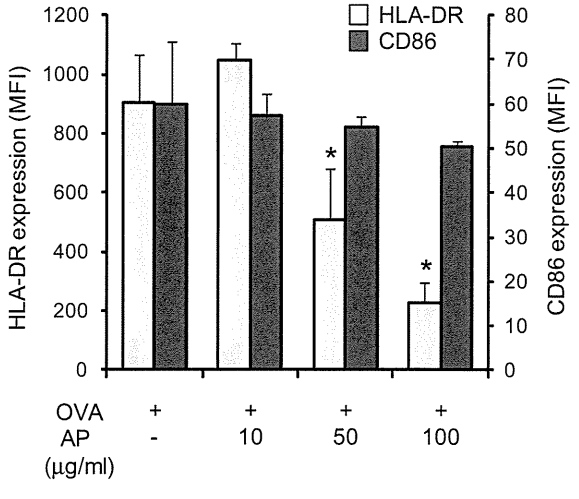

Fig. 3. Suppressive effects of apple polyphenol extract (AP) on the expression of HLA-DR and CD86 in OVA-stimulated THP-1-derived human dendritic cells (TDDCs). TDDCs were treated with AP (10, 50, and 100 μg/ml) in the presence of OVA for 72 h. Data are presented as means ± SD of three independent experiments.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

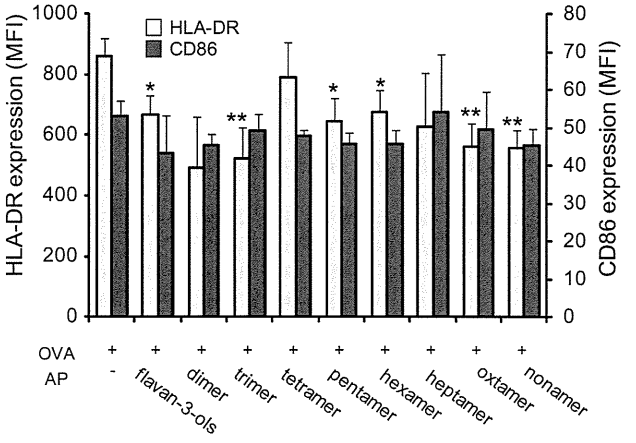

Fig. 4. Suppressive effects of the fractionated procyanidins on expression of HLA-DR and CD86 in OVA-stimulated TDDCs. TDDCs were treated with fractionated procyanidins (50 μg/ml) in the presence of OVA for 72 h. Data are presented as means ± SD of two independent experiments. $^{*}p < 0.05$ and $^{**}p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

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

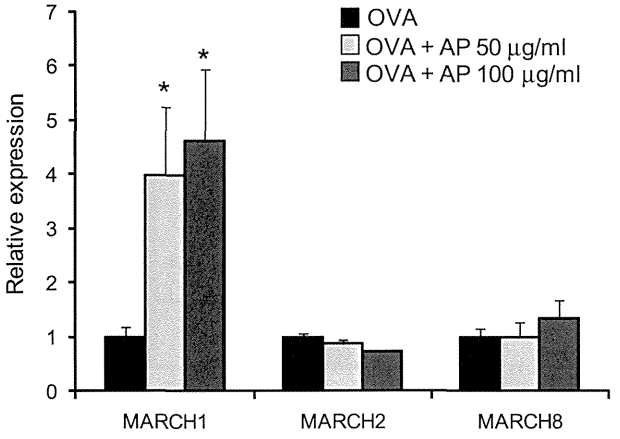

Fig. 5. Effect of AP on membrane-associated RING-CH (MARCH) family gene expression of OVA-stimulated TDDCs. TDDCs were treated with AP (50 and 100 μg/ml) in the presence of OVA for 72 h. The gene expression levels of MARCH1, 2, and 8 were quantified by real-time PCR. Data are presented as means ± SD 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-1β secretion level among the AP and oligomer-treated cells (Fig. 6A). Particularly, significant 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 & Lehner, 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-1β 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-1β 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

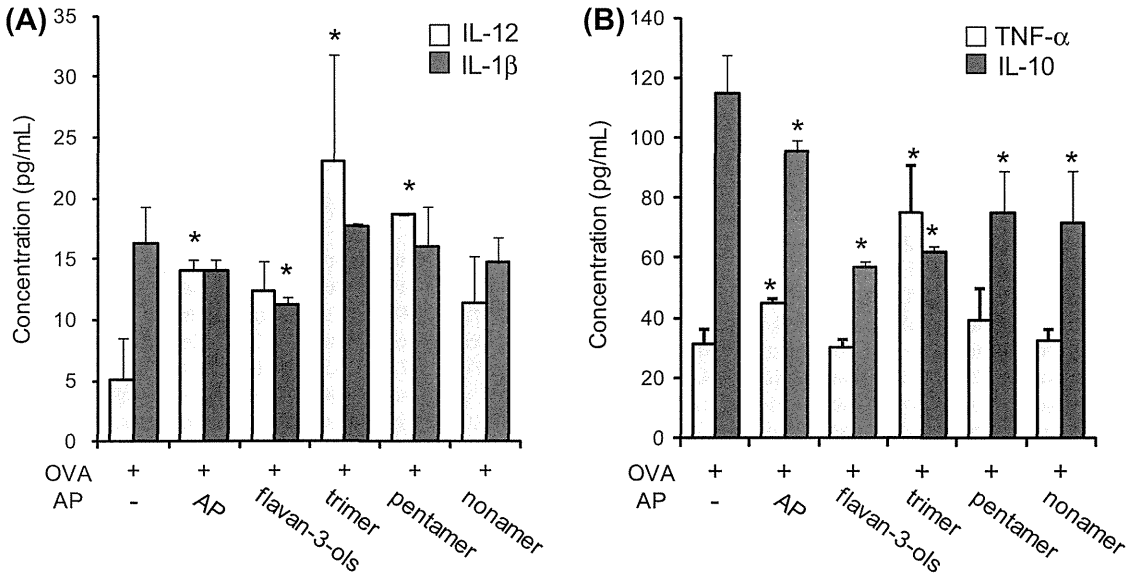

Fig. 6. Changes in secretion profiles of IL-12 and IL-1β (A), TNF-α and IL-10 (B) in OVA-stimulated TDDCs by AP treatment. TDDCs were treated with AP and the fractionated procyanidins (50 μg/ml) in the presence of OVA for 72 h. The culture supernatant was measured for IL-12, IL-1β, TNF-α, and IL-10 secretion by ELISA. Data are presented as means ± 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–FcεRI 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.

Acknowledgements

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Article

Immunological Effects of Oenothein B, an Ellagitannin Dimer, on Dendritic Cells

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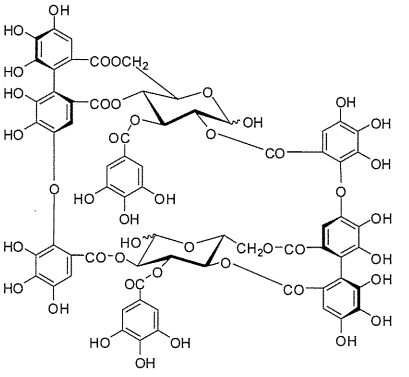
Abstract: Oenothein B is a unique macrocyclic ellagitannin dimer that has been found in various medicinal plants belonging to Onagraceae, Lythraceae, and Myrtaceae, with diverse biological activities. The immunological effects of tannins in terms of cytokine-release from macrophages and monocytes have been discussed, while the effects on other immunocompetent cells have been the subject of minimal investigation. We evaluated the immunomodulatory effects induced by tannin treatment in human dendritic cells (DCs), which play a critical role in the initial immune response, by measuring the changes in cytokine production, cell differentiation, and cell viability. Oenothein B showed significant down-regulation of the expression of cell surface molecules, CD1a and CD83, suggesting the inhibition of DC differentiation and/or maturation. The suppressive effect on DCs was associated with the induction of apoptosis without the activation of caspase-3/7, 8, and 9, and this was supported by the morphological features indicating significant nuclear condensation. Oenothein B also markedly suppressed the production of inflammatory cytokines, such as IL-1β and IL-6, in a dose-dependent manner. These data may, in part, be able to explain the traditional use of tannin-containing medicinal plants for the treatment of a variety of inflammatory diseases, including inflammatory bowel disease, celiac disease, and rheumatoid arthritis.

Keywords: dendritic cell; oenothein B; epigallocatechin gallate; cytokine; caspase

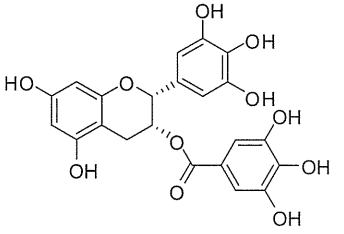
1. Introduction

Polyphenols, widely distributed in medicinal plants, foods and beverages, have currently been attracting great interest because of their diverse biological properties, including potent antioxidative effects, which are beneficial to human health. Among such bioactive polyphenols are ellagitannins, classified as large molecular weight tannins and found in many traditional medicines in Japan and China, and which have been demonstrated to exhibit inhibitory effects on various enzymes, anti-tumor and antimicrobial activities, as well as antioxidative effects [1]. The biological activities of tannins also include a number of effects associated with immunomodulation, such as host-mediated antitumor effects by oenothein B and other dimeric ellagitannins [2], anti-leishmanial activity by geraniin and other dehydroellagitannins [3] and antitumor-promoting effects by geraniin and a green tea tannin, EGCG [4].

Figure 1. Structures of oenothein B and EGCG.



(oenothein B)



(−)-epigallocatechin gallate (EGCG))

Among tannins, oenothein B is a unique macrocyclic ellagitannin dimer (Figure 1) that was first isolated from *Oenothera erythrosepala* (Onagraceae) [5] and later found widely distributed in various medicinal plants belonging to Onagraceae, Lythraceae [6] and Myrtaceae [7]. Notably its host-mediated antitumor activity against a model cancer cell line, sarcoma 180 was the most potent among over a hundred tannins and related polyphenols examined. This effect was attributed to the activation of macrophage-releasing IL-1β [2]. Meanwhile, the suppressing effect of IL-1β and IL-6 secretion by oenothein B associated with anti-inflammatory effects was also reported in recent study [8]. Other immunomodulatory effects of tannins have also been discussed, mostly in terms of

cytokine-release from macrophages and monocytes [9], while the effects of tannins on other immunocompetent cells have been the subject of minimal investigation to date.

Among the immune cells, DCs are bone marrow-derived leucocytes and play a critical role in the initial immune response. Immature DCs have strong phagocytic ability, and after antigen uptake and maturation, matured DCs migrate to lymph nodes and present antigens to naive T cells. DCs, macrophages and B cells are involved in antigen presentation with MHC class II molecules and therefore these cells are called professional antigen-presenting cells. Another important function of cytokine production inherent to DCs is its constitutive role in initiating inflammation related to some autoimmune diseases, including inflammatory bowel disease, celiac disease [10] and rheumatoid arthritis [11].

In the present study, we assessed the effects of oenothein B on human DCs by measuring changes in cytokine production, cell differentiation, and cell viability, thereby enhancing our understanding of tannins in medicinal plants.

2. Results

2.1. Analysis of Cell Apoptosis Using Flow-Cytometry

EGCG is known to induce cell death in an apoptotic (≤50 μM) or necrotic (>50 μM) manner [12]. To evaluate the effects of oenothein B on DCs, we analyzed the cells stained with PI and Annexin V using flow-cytometry. The cells stained with PI were taken as dead cells (Figure 2A), and PI (−) and annexin V (+) cells were considered as apoptotic cells (Figure 2B).

Figure 2. Identification of the type of cell death. iDCs were seeded at a density of 3.0×10^5 cells per well in 12-well plates, and cultured with EGCG (100 μM), oenothein B (25, 100 μM) or culture medium (blank) for 22 h supplemented with TNF-α (75 ng/mL) and LPS (100 ng/mL). (**A**) Cells were harvested and stained with propidium iodide (PI) before flow-cytometric analyses. PI-stained cells were increased with tannin treatment and the cells were presumed to be necrotic cells; (**B**) Cells were stained with PI and annexin V, and analyzed by flow-cytometry. Apoptotic ratio of DCs was estimated by double-staining with PI and annexin V. Each data point represents the mean ± SD, $n = 3$, for * $p < 0.05$, ** $p < 0.01$ *vs.* blank group (□) using the student's *t*-test.

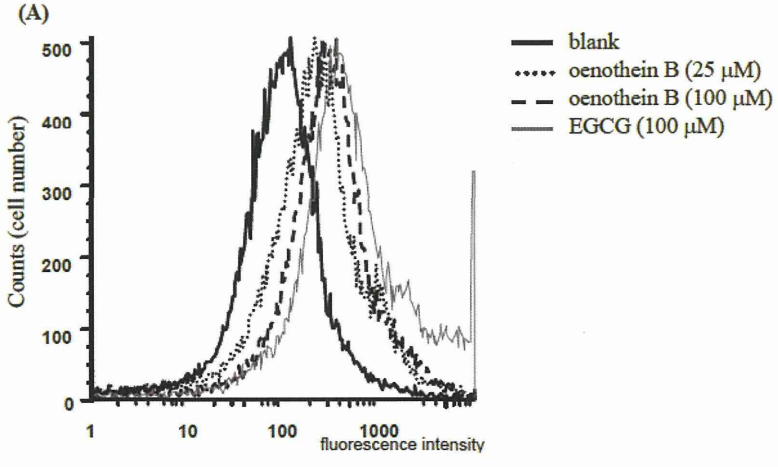


Figure 2. *Cont.*

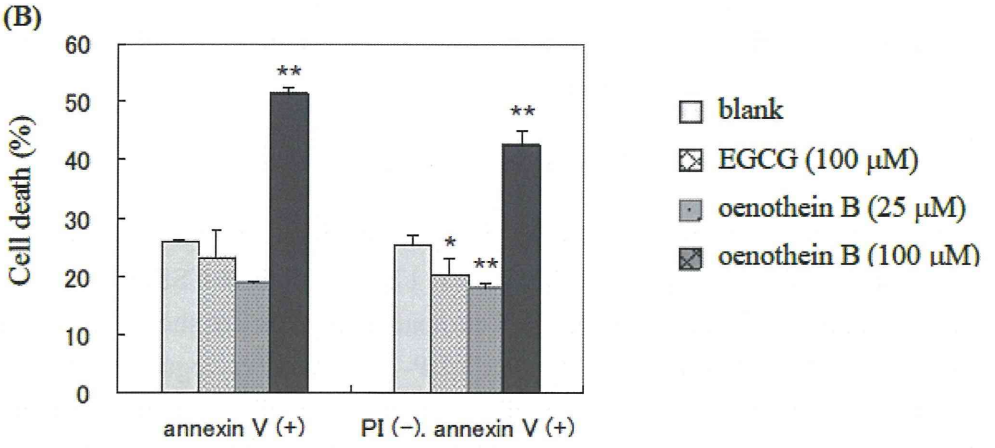
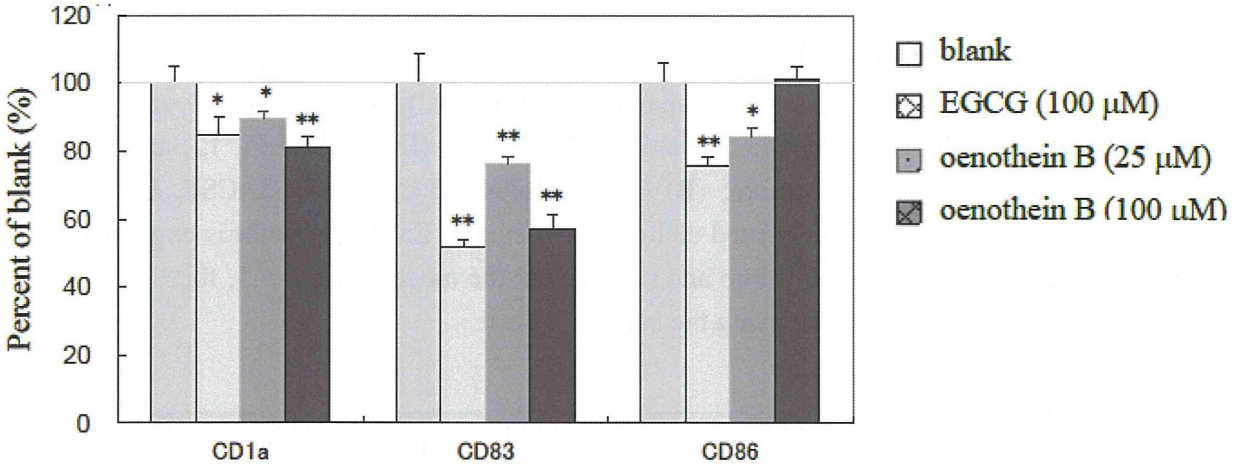


Figure 3. Changes in the expression of cell surface molecules. iDCs were cultured with EGCG (100 μM), oenothein B (25, 100 μM) or culture medium (blank) for 22 h at a density of 3.0×10^5 cells per well in 12-well plates supplemented with TNF-α (75 ng/mL) and LPS (100 ng/mL). Cells were treated with fluorescence-labeled monoclonal antibodies against CD1a, CD83 and CD86 for flow-cytometric analysis, and the expression of cell surface molecules were determined. Each data point represents the mean ± SD, $n = 3$, for * $p < 0.05$, ** $p < 0.01$ *vs.* blank group (□) using the student's *t*-test.



As shown in Figures 2 and 3, treatment with 100 μM oenothein B specifically induced the cell death and apoptosis of cultured DCs, whereas EGCG-treatment (100 μM) did not induce apoptosis to a greater extent than that of control. On the other hand, low-dose treatment (25 μM) of oenothein B reduced the number of apoptotic cells, although the ratio of total PI (+) cells was slightly higher than that of the non-treatment group.

2.2. Flow-Cytometric Analyses of Cell Surface Molecules

The cultured cells were stained with fluorescence-labeled monoclonal antibodies against CD1a, CD83, CD86, and analyzed using flow-cytometry. The cell surface molecules of CD1a and CD83 were

down-regulated by oenothein B or EGCG, while CD86 was not significantly changed (Figure 3) upon treatment with oenothein B (100 μM). As CD83 expression is known to be a marker of matured DCs [13] and to play a critical role in antigen presentation, that the tannins studied implied that tannins are inhibitors of iDC differentiation and/or maturation.

2.3. Quantification of Cytokines in Cell Culture Medium Supernatant

The cell culture supernatants were analyzed using a Bio-Plex multiple suspension array kit to quantify 17 cytokines (IL-1β, 2, 4, 5, 6, 7, 8, 10, 12, 13, 17, G-CSF, GM-CSF, IFN-γ, MCP-1, MIP-1β and TNF-α). The parameters significantly changed compared to blank are shown in Figure 4. Cytokine productions of IL-1β, IL-6, IL-12, IL-17, IFN- γ and MIP-1β were down-regulated in a dose-dependent manner by oenothein B-treatment, and the efficacy of oenothein B was more potent than that of EGCG. Furthermore, the inflammatory cytokines IL-1β and IL-6 were significantly down-regulated. These results suggest that these tannins may have anti-inflammatory effects through the inhibitory effects of DC inflammatory cytokine production.

Figure 4. Cytokine production upon treatment with tannins. Cells were cultured in the presence of EGCG (100 μM), oenothein B (25, 100 μM) or culture medium (blank) at a density of 1.4×10^5 cells per well in 12-well plates for 22 h supplemented with TNF-α (75 ng/mL) and LPS (100 ng/mL). Each cultured medium was collected and applied to a Bio-Plex multiple suspension array kit (Bio-Rad) for cytokine quantification [IL-1β, 2, 4, 5, 6, 7, 8, 10, 12, 13, 17, granulocyte colony stimulating factor (G-CSF), granulocyte macrophage colony stimulating factor (GM-CSF), interferon (IFN)-γ, monocyte chemotactic protein (MCP)-1, macrophage inflammatory protein (MIP)-1β and tumor necrosis factor (TNF)-α]. (**A**) Quantification of cytokines (IL-1β, 2, 4, 6, 12, and 17) in tannin-treated cell culture medium; (**B**) Quantification of cytokines (G-CSF, GM-CSF, IFN-γ, and MIP-1β) in tannin-treated cell culture medium. Each data point is expressed as percentage relative with blank group and represented the mean ± SD, $n = 3$, for * $p < 0.05$, ** $p < 0.01$ *vs.* blank group (□) using the student's *t*-test.

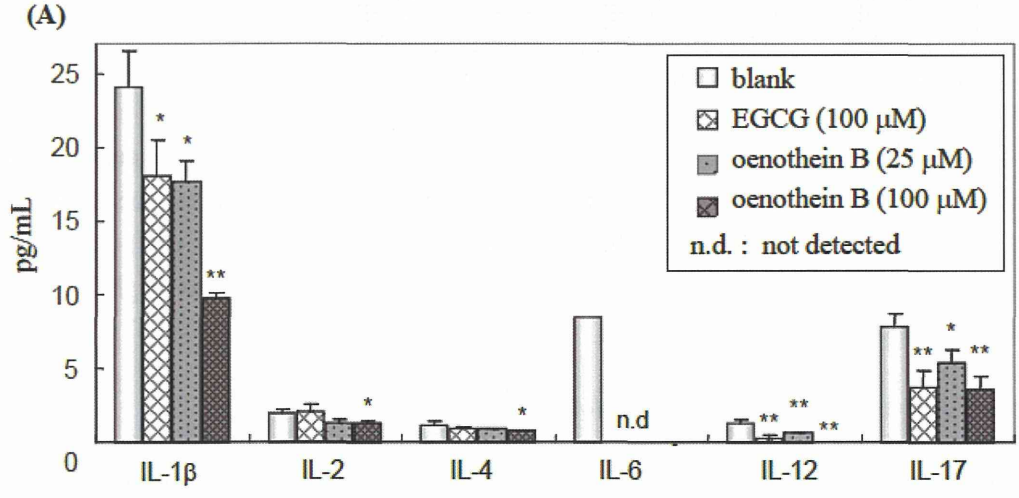
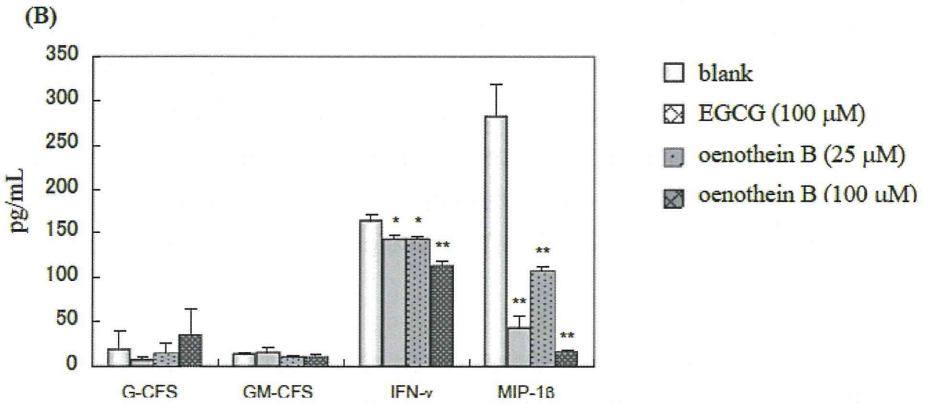


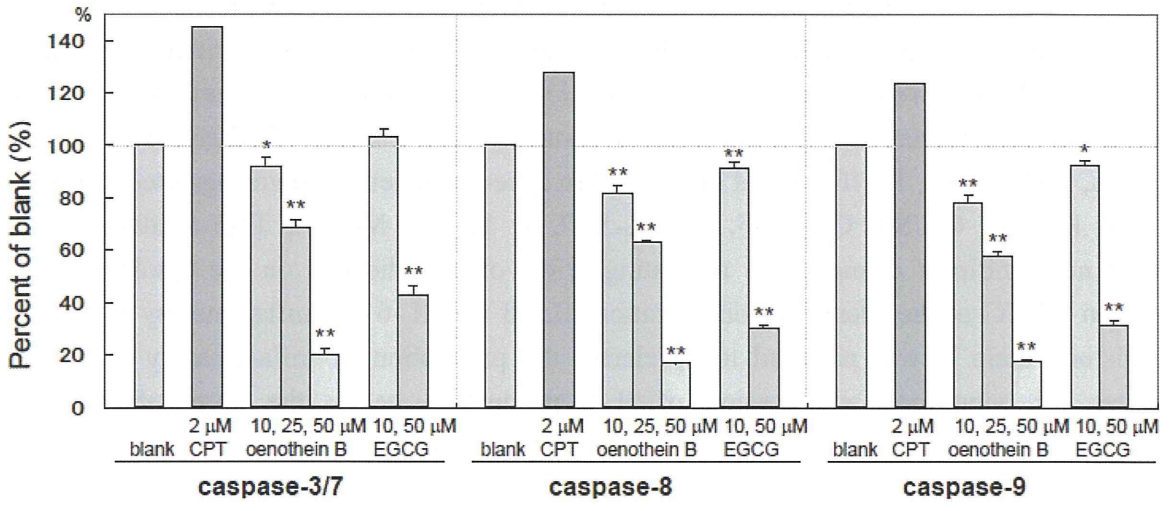
Figure 4. *Cont.*



2.4. Measurement of Caspase Activities

Caspases play an important role in cell apoptosis and are composed of several sub-types with individual functions. Caspase-3 and 7 are known to act as effectors of apoptosis and caspase-8 and 9 are initiators of apoptosis. As shown in Figure 5, caspase-3/7, 8 and 9 activities of cultured DCs supplemented with oenothein B or EGCG were dose-dependently and significantly down-regulated. While CPT, which is a well-known apoptosis-inducing compound, activated these enzymes as anticipated. These results show that these tannins preferentially inhibit the activation of caspase-3/7, 8 and 9. The findings suggest that induction of cell death with tannin treatment occurred in dose-dependent and caspase-independent manners.

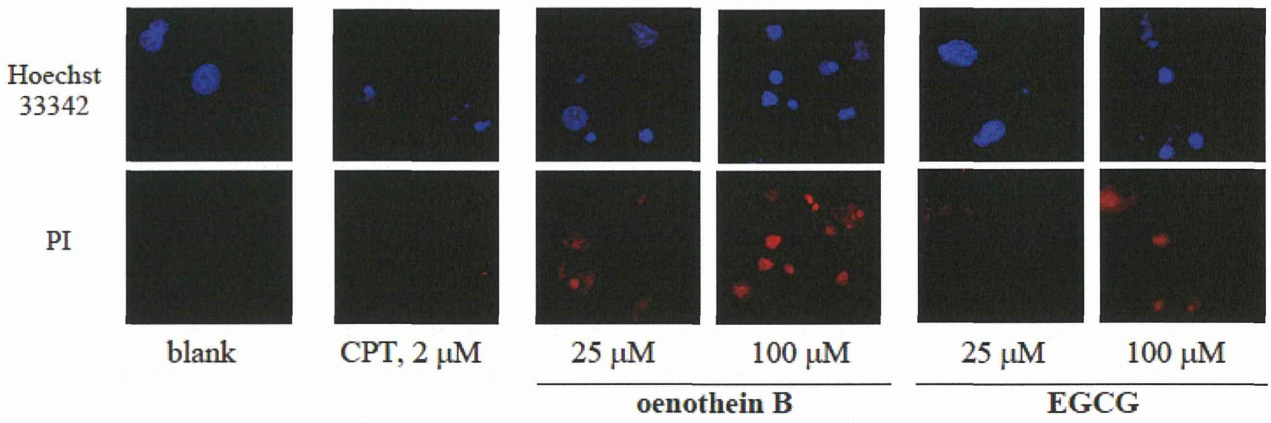
Figure 5. Caspase activities in tannin-treated DCs. Cells were cultured at a density of 2.3×10^5 cells per well in 12-well plates for 22 h in the presence of camptothecin (CPT), EGCG (10, 50 μM), oenothein B (10, 25, 50 μM) or culture medium (blank) supplemented with TNF-α (75 ng/mL) and LPS (100 ng/mL). Each cell lysate was prepared using Reporter Lysis Buffer (Promega), and the caspase-3/7, -8 and -9 activities were measured by the respective caspase assay kits (Promega). Each data point is expressed as percentage relative with blank group and represented the mean ± S.D., $n = 3$, for * $p < 0.05$, ** $p < 0.01$ *vs.* blank group (□) using the student's *t*-test.



2.5. Morphological Analysis of Tannin-Treated DCs

Cultured DCs were stained with PI and Hoechst 33342, and nuclear fragmentation or other features were evaluated under a fluorescence microscope (×400). DCs treated with oenothein B or EGCG showed significant reduction of nuclear size, in contrast with cell nuclear fragmentation in the CPT-treated group as positive control (Figure 6). These morphological features resemble AIF (apoptosis-inducing factor)/PARP (poly (ADP-ribose) polymerase)-dependent cell death [14]. These results suggest that oenothein B and EGCG affect nuclear size, not cell nuclear fragmentation as observed with CPT.

Figure 6. Fluorescence microscopy observations of tannin-treated DCs. Cultured cells (3.3×10^5 cells per well in 12-well plates for 24 h) treated with EGCG (25, 100 μM), oenothein B (25, 100 μM) or culture medium (blank) were fixed to CELL-TAK (BD Biosciences) coated 8-well chamber slides. After staining with Hoechst 33342 and PI, cells were mounted with ProLong Gold Antifade Reagent (Invitrogen, Carlsbad, CA, USA) and evaluated under a fluorescence microscope (×400).



3. Discussion

We investigated the influence of tannins on DC associated immune responses, and found that oenothein B and EGCG have immunoregulatory effects on DCs through suppression of cell surface molecules, down-regulation of cytokine production and induction of their apoptosis.

The inhibitory effect of these tannins on the expression of cell surface molecules CD1a, CD83 and CD86 indicated they would induce the dysfunction of DC-mediated immune responses by the inhibition of cell maturation and subsequent antigen presentation. Furthermore, for cytokine production, IL-1β, 6, 12, 17, IFN-γ and MIP-1β were dose-dependently down-regulated, whereas other cytokines, IL-2, 4, G-CSF, GM-CSF, and IL-5, 7, 8, 10, 13, MCP-1, TNF-α (data not shown), displayed no significant change. The regulating effect of oenothein B on the cytokines was more potent than EGCG at the identical concentration. IL-1β and IL-6 are inflammatory cytokines [15]. Although oenothein B was reported to accelerate the production of inflammatory cytokines from monocytes [9], significant suppression of IL-6 production below the level of detection and down-regulation of IL-1β might thus induce the anti-inflammatory effect of tannins through DCs.

For cell viability evaluated by flow-cytometric assays, the higher increase in amount of PI stained cells in the tannin-treated groups indicated that tannins induced cell death. Though the increase in PI (−) and annexin V (+) cells upon treatment with a high concentration of oenothein B (100 μM) indicated potent induction of cell apoptosis, oenothein B (25 μM) and EGCG (100 μM) showed less severe apoptotic features. To investigate the mechanism of cell death, we examined the effects on caspases (caspase-3/7, 8 and 9), which play crucial roles in cell apoptosis. Caspase-3/7 and 8/9 are known to act as effectors and initiators of apoptosis, respectively. As shown in Figure 5, none of these caspases were activated but, instead, down-regulated dose-dependently by the tannins, suggesting that the apoptosis mechanisms were not caspase-dependent. Though EGCG was reported to induce caspase-dependent apoptosis in normal rat kidney interstitial fibroblast cells at a low concentration (≤50 μM) [12], unexpectedly, it showed no caspase activation of DCs (Figure 5) in our study. The mechanisms of induced-apoptosis with or without caspase thus appear to differ according to cell type. The caspase-independent apoptosis in tannin-treated DCs might be related to morphological changes showing nuclear condensation without DNA fragmentation, which is a characteristic feature of caspase-dependent apoptosis (Figure 6). In addition, necrosis-like cell expansion and DNA smearing were not observed in the tannin-treated cells. The findings suggest that these features are similar to AIF/PARP-dependent cell apoptosis. Further multidirectional approaches to elucidating their mechanisms should be investigated.

4. Experimental Section

4.1. Cell Line, Chemicals and Biochemicals

EGCG was purchased from Nakahara Kagaku (Gifu, Japan). Purified LPS (*Escherichia coli*; serotype O26: B6) was obtained from Sigma (St. Louis, MO, USA) and dissolved in endotoxin-free water. iDCs and culture medium ACS-100 were purchased from NEMOD GmbH & Co. (Berlin, Germany), and all cultures were performed in ACS-100. Recombinant human TNF-α and DNase I were obtained from PeproTech Inc. (Rocky Hill, NJ, USA). PI, annexin V, fluorescence (APC, FITC, PE)-labeled monoclonal antibodies (to CD1a, 83, 86) and CELL-TAK were purchased from BD Biosciences (Two Oak Park, MA, USA). CPT, Triton X-100 and paraformaldehyde phosphate buffer were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Hoechst 33342 and PI for nuclear staining were purchased from Molecular Probes (Eugene, OR, USA). Prolong Gold Antifade Reagent was obtained from Invitrogen (Carlsbad, CA, USA). Oenothein B was isolated from the leaves of *Eucalyptus globulus* as reported previously [16].

4.2. Cell Culture

Frozen iDCs were rapidly thawed in a water bath (37 °C), and culture medium composed of ACS-100 with DNase I (20 U/mL) was added to a final volume of 50 mL. After centrifugation (7 min, 200 × *g*) and removal of the culture medium, cells were re-suspended in ACS-100 supplemented with TNF-α (75 ng/mL) and LPS (100 ng/mL). Cells were further cultured at a density of 3.0×10^5 cells per well in 12-well plates, supplemented with EGCG (10, 50, 100 μM), oenothein B (10, 25, 50, 100 μM) or culture medium (blank), for 22 h at 37 °C in a 5% CO_2 air environment. For measurements of

caspase activity and morphological changes, cells were cultured for 24 h using CPT (2 μM) as a positive control. After culturing, cells were collected and used for flow cytometric analyses, measurement of caspase activities and fluorescence microscopy (Olympus IX71, Olympus, Tokyo, Japan). The supernatants were used for cytokine assays.

4.3. Flow Cytometric Analyses

Cultured iDCs were collected and stained with PI and annexin V (for analysis of cell apoptosis) or fluorescence (APC, FITC, PE)-labeled monoclonal antibodies (to CD1a, 83, 86, for analysis of cell surface molecules) at concentrations indicated by the manufacturers. Data were collected using a FACSCalibur flow cytometer (Becton Dickinson, and Company, Franklin Lakes, NJ, USA) and analyzed with BD CellQuestTM software.

4.4. Quantification of Cytokines

Cell culture medium supernatants were analyzed using a Bio-Plex multiple suspension array kit (Bio-Rad Laboratories, Hercules, CA, USA) and 17 cytokines were quantified [IL-1β, 2, 4, 5, 6, 7, 8, 10, 12, 13, 17, G-CSF, GM-CSF, IFN-γ, MCP-1, MIP-1β and TNF-α]. Data were collected and analyzed using a Bio-Plex suspension array system.

4.5. Measurement of Caspase Activities

Caspase activities were assayed using a Apo-ONE Homogeneous caspase-3/7 kit, Caspase-Glo 8 assay kit and Caspase-Glo 9 assay kit (Promega, Madison, WI, USA). Reporter Lysis Buffer (Promega, Madison, WI, USA) was added to the cultured cells and freeze-thawed for cell lysis, and the homogenates were used for measurement of caspase activities. Each cell lysate was transferred to a 96-well microplate and Apo-ONE Homogeneous Caspase-3/7 Reagent (Promega, Madison, WI, USA) or Caspase-Glo 8 (or 9) Reagent (Promega) was added. After incubation at room temperature for 1 h, fluorescence for Caspase-3/7 and light emissions for Caspase-8 or 9 were measured according to the methods indicated by the manufacturers.

4.6. Observation of Morphological Changes

Cultured cells were collected and washed twice with PBS (−), and transferred to a CELL-TAK coated 8-well culture slide and left for 1 h to allow for cell adhesion. After removal of PBS (−), the cells were fixed with 4% paraformaldehyde for 30 min, and permeabilized with 0.2% Triton X-100 for 10 min.

After blocking with 2% BSA, the cells were stained with PI and Hoechst 33342. Finally, air-dried slides were mounted with ProLong Gold Antifade Reagent. Each slide was evaluated under a fluorescence microscope (×400).

5. Conclusions

The present study demonstrated that tannins exert peripheral anti-inflammatory effects by down-regulation of cytokines and induction of dysfunction and apoptosis in DCs. These effects might

be important especially in digestive organs, since tannins are generally considered to be stable in acidic conditions and thus could travel without modification through the pharyngeal tube and stomach until their metabolism in the small intestine [17]. These data may, in part, be able to explain the traditional use of tannin-containing medicinal plants for the treatment of a variety of inflammatory diseases.

Conflict of Interest

The authors declare no conflict of interest.

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