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Original article

Caffeic amide derivatives inhibit allergen-induced bone marrow-derived dendritic cell maturation

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ABSTRACT

Background: Caffeic amides are derivatives of caffeic acid, which have antioxidant and anti-inflammatory properties, and high *in vivo* stability. The therapeutic effect of caffeic amides on allergic diseases, and especially on the maturation of bone marrow-derived dendritic cells (BM-DCs), remains unclear. In this study, we investigated the therapeutic potential of caffeic amides on allergic diseases by evaluating the maturation of DCs and evaluated their potential in inducing the differentiation of T_H2 cells.
Methods: BM-DCs isolated from BALB/c mice were treated with different caffeic amide derivatives for 48 h and the expression of surface markers was analyzed by flow cytometry. The differentiation of $CD4^+$ T cells was detected by the 5-bromo-2-deoxyuridine (BrdU) incorporation assay and cytokine production was analyzed by ELISA.
Results: Our results showed that among the six caffeic amides tested herein, only 36 M significantly inhibited the antigen-induced maturation of DCs associated with the expression of CD80, CD86, and major histocompatibility complex II (VC ovalbumin (OVA)+ thymic stromal lymphopoietin (TSLP) *vs.* 36 M OVA + TSLP). Additionally, the isolation and co-culture of antigen-specific $CD4^+$ T cells with 36 M-treated BM-DCs suppressed the antigen-specific differentiation of T_H2 cells.
Conclusion: Among the six caffeic amides tested herein, 36 M (*N*-octyl caffeamide) might possess therapeutic potential for allergic diseases.
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Introduction

Caffeic acid is a phenolic compound that is abundantly found in edible plants [1]. It has been extensively studied owing to its antioxidant potential, which is attributable to its free-radical scavenging properties, along with its ability to donate hydrogen atoms or electrons, and chelate metal cations [2]. Caffeic acid also exhibits *in vitro* and *in vivo* anti-inflammatory effects by inhibiting the activities of 5-lipoxygenase and 12-lipoxygenase [3], and the lipopolysaccharide-induced production of nitric oxide and prostaglandin E2 in macrophages [1]. It has also been reported that caffeic acid suppresses HCl/ethanol-induced gastric inflammation in mice [4]. In addition to its anti-inflammatory activities, caffeic acid also possesses anticancer [5,6], antiviral, and anti-diabetic properties that are possibly associated with its antioxidant activity [5] and suppression of critical transcription factors, including nuclear factor of activated T cells, nuclear factor kappa B, and activator protein 1 [4]. Similar to caffeic acid, caffeic acid derivatives also exhibit antioxidant, anti-inflammatory, and anticancer properties, with the most well-studied caffeic acid derivative being caffeic acid ester [3]. However, the use of these caffeic acid esters is restricted by their low metabolic stability [7]. In this study, we therefore attempted to synthesize caffeic acid amides and investigate their anti-allergic activities in murine bone marrow-derived dendritic cells (BM-DCs).

The prevalence of allergic diseases, including asthma, rhinoconjunctivitis, sinusitis, food allergies, atopic dermatitis, angioedema, urticaria, and anaphylaxis, has dramatically increased in recent decades in both developing and developed countries [8].

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Several risk factors, including genetic factors, exposure to allergens, and indoor and outdoor pollution contribute to the onset of allergic diseases [9]. Allergic diseases are caused by immune dysregulation that is mediated by the expansion of $CD4^+$ T helper 2 (T_H2) cells, along with the isotype switching of B cells to generate IgE antibodies specific for common environmental allergens. The T_H2 cells induce allergic inflammation *via* the release of cytokines, including interleukin (IL)-4, IL-13, IL-5, and IL-9 that regulate the synthesis of IgE, airway hyper-responsiveness, eosinophil proliferation, and mast cell proliferation, respectively [10]. DCs are essential for inducing the activation and differentiation of naïve $CD4^+$ T cells, as well as effector $CD4^+$ T cells, including T_H2 cells, in allergic diseases [2,11]. Since DCs are important for inducing the immune response during ongoing inflammation in the lungs, nose, and skin, interfering with the function of DCs could offer an effective strategy for the treatment of allergic diseases. Moreover, the altered maturation of DCs could help evade the sensitization of T_H2 cells, which could possibly offer extended therapeutic effects.

In this study, we investigated the therapeutic potential of caffeic amides in allergic diseases by evaluating their effect on the maturation of DCs and their potential in inducing the differentiation of T_H2 cells. We observed that among the six caffeic amides tested herein, only 36 M could significantly inhibit the antigen-induced maturation of DCs and differentiation of T_H2 cells.

Materials and methods

Reagents and chemicals

Ovalbumin (OVA; grade V) was purchased from Sigma-Aldrich (St. Louis, MO, USA). RPMI-1640 medium, Hank's balanced salt solution (HBSS), penicillin, streptomycin, L-glutamine, and fetal bovine serum (FBS) were purchased from Invitrogen (Carlsbad, CA, USA). Thymic stromal lymphopoietin (TSLP) was purchased from R&D Systems (Minneapolis, MN, USA).

Synthesis of caffeic amide derivatives

The compounds considered in this study were synthesized according to the methods described hereafter. Caffeic acid (100 mg; 0.56 mmol) was dissolved in a mixture of 1 mL *N*, *N*-dimethylformamide and 80 μL triethylamine. This was followed by the addition of 80 μL (1.2 eq.) of the amine derivatives to the reaction solution and subsequent cooling in an ice bath (0 °C). Benzotriazol-1-yl-oxy-tris(dimethylamino)phosphonium hexafluorophosphate (295 mg; 1.2 eq.), dissolved in 5 mL CH_2Cl_2, was then added to the reaction mixture with constant stirring for 30 min (Fig. 1A). The reaction mixture was then allowed to cool to room temperature, where it was kept for 2 h. The solvent was subsequently evaporated under vacuum conditions and the crude mixture was partitioned with ethyl acetate and H_2O. The organic layer was washed with 3 N aqueous HCl, followed by washing with 10% aqueous $NaHCO_3$ solution. The products were purified by silica gel chromatography, with the yields of 36, 36H, 36 M, 36-6, 36-16, and 36-25B ranging from 70% to 80%. A Bruker Avance 500 spectrometer (Bruker, Billerica, MA, USA) was used to record the ^1H and ^{13}C nuclear magnetic resonance (NMR) spectra, while the electron impact mass spectra were determined by a Finnigan TSQ-46C mass spectrometer (Finnigan MAT, Inc., San Jose, CA, USA). The infrared spectra were recorded on a Nicolet Magna-IR 550 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The structures of the compounds considered in this study are depicted in Table 1. The caffeic amide derivatives synthesized herein were stored for one year at 4 °C.

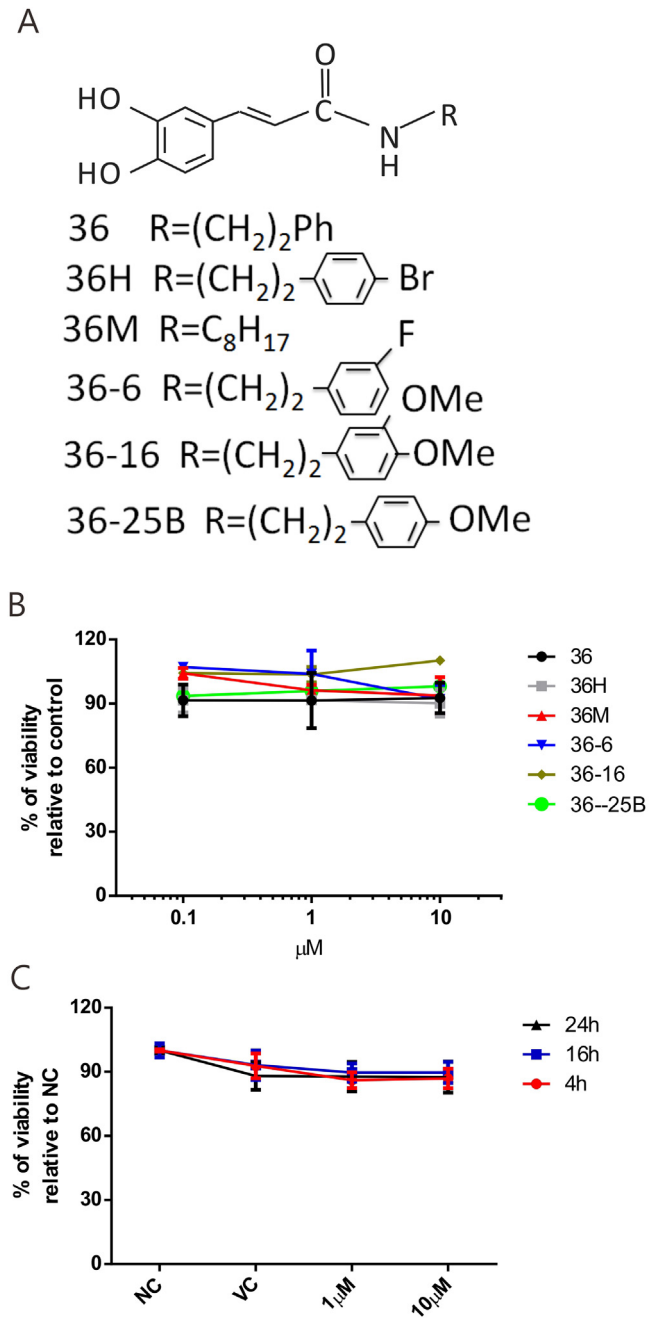
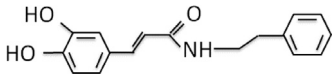
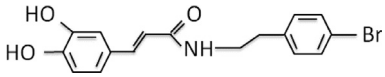
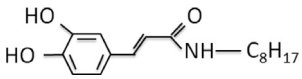
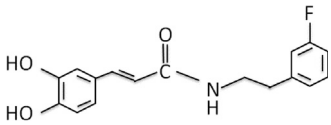
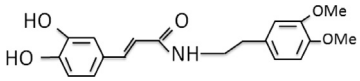
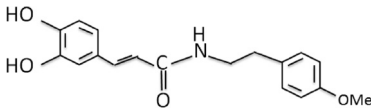


Fig. 1. Synthesis and cytotoxicity of caffeic amide derivatives. (A) Synthesis of caffeic amide derivatives, as described in the methods section. (B) Murine BM-DCs (5×10^4 cells) were cultured in 96-well plates and treated with caffeic amides for 48 h. The cytotoxicity was evaluated by the MTT assay as described in the methods section. (C) Murine BM-DCs (5×10^5 cells) were cultured in 96-well plates and treated with 36 M for 4, 16, and 24 h. The cytotoxicity was evaluated by the MTT assay. The data are expressed as the mean ± SD ($n \geq 3$). $^*p < 0.05$, compared to the vehicle control (VC; 0.1% DMSO).

Generation of murine BM-DCs

The BM-DCs were prepared according to a method previously described, with some modifications [1,12,13]. Female BALB/c mice, aged between 3 and 5 weeks, were purchased from the National Laboratory Animal Center, National Research Laboratories, Taiwan. The mice were sacrificed by exposure to CO_2. The animal experiment protocols were approved by China Medical University (approval number: 103-128-N). Briefly, the BM cells from the femurs and tibiae of BALB/c mice were first depleted of red blood

Table 1
Structure and NMR of caffeic acid amides derivatives.

36		mp: 148-149°C; IR νmax (cm-1): 3288, 1642, 1591, 1523, 1361, 1279, 1036, 975, 849. 1H NMR (CD3COCD3, 500 MHz): δ 2.8 (2H, t, J = 6.8 Hz), 3.5 (2H, q, J = 6.8 Hz), 6.4 (1H, d, J = 15.2 Hz), 6.8 (1H, d, J = 8.1 Hz), 6.9 (1H, dd, J = 8.1, 1.8 Hz), 7.1 (1H, d, J = 1.8 Hz), 7.2-7.30 (5H, m), 7.4 (-NH, br. s), 7.4 (1H, d, J = 15.2 Hz), 8.2 (-OH, s), 8.4 (-OH, s). EI-MS m/z (%): 283 (M+, 17), 178 (22), 163 (100)UV(MeOH) λmax (logε): 322(4.4), 296(4.4), 245(4.3), 216(4.6)nm.
36H		mp: 198-199°C; IR νmax (cm-1): 3317, 1653, 1593, 1527, 1487, 1441, 1367, 1285, 1195, 1116, 1016, 988, 811. 1H NMR (CD3COCD3, 500 MHz): δ 2.8 (2H, t, J = 6.6 Hz), 3.5 (2H, q, J = 6.6 Hz), 6.4 (1H, d, J = 16.0 Hz), 6.8 (1H, d, J = 8.0 Hz), 6.9 (1H, dd, J = 8.0, 1.8 Hz), 7.1 (1H, d, J = 1.8 Hz), 7.2 (2H, d, J = 8.0 Hz), 7.3 (-NH, br. s), 7.4 (1H, d, J = 16.0 Hz), 7.4 (2H, d, J = 8.0 Hz), 8.1 (-OH, s), 8.3 (-OH, s). EI-MS m/z (%): 361 (M+, 8), 207 (15), 178 (29), 163 (100), 89 (24)UV(MeOH) λmax (logε): 322(4.4), 295(4.3), 240(4.4), 219(4.5)nm.
36 M		mp: 111-112°C; IR νmax (cm-1): 3286, 1642, 1588, 1520,1363, 1277, 1112, 975, 811. 1H NMR (CD3COCD3, 400 MHz): δ 0.84 (3H, t, J = 6.6 Hz), 1.2 (10H, m), 1.5 (2H, quin, J = 6.6 Hz), 3.3 (2H, q, J = 6.6 Hz), 6.5, 7.4 (each 1H, d, J = 15.6 Hz), 6.82 (1H, d, J = 8.2 Hz), 6.9 (1H, dd, J = 8.2, 1.8 Hz), 7.1 (1H, d, J = 1.8 Hz). EI-MS m/z (%): 291 (M+, 18), 220 (8), 193 (11), 178 (31), 163 (100), 145 (8), 135 (13), 128 (22), 117 (11), 98 (8), 89 (19), 84 (12)UV(MeOH) λmax (logε): 322(4.3), 294(4.3), 238(3.9), 219(4.4)nm.
36-6		mp: 155-157 °C; IR νmax (cm-1): 3503, 3352, 3179, 1647, 1589, 1535, 1489, 1443, 1361, 1304, 1273, 1121, 1184, 968. 1H NMR (CD3OD, 500 MHz): δ 7.4, 6.3 (1H each, d, J = 15.6 Hz), 7.3 (1H, td, J = 7.9, 6.2 Hz), 7.1 (1H, d, J = 7.7 Hz), 7.0 (1H, d, J = 2.0 Hz), 7.0 (1H, brd, J = 9.9 Hz), 6.9 (1H, td, J = 8.3, 2.5 Hz), 6.9 (1H, dd, J = 8.1, 2.0 Hz), 6.8 (1H, d, J = 8.1 Hz), 3.5, 2.8 (2H each, t, J = 7.2 Hz) ; UV(MeOH) λmax (logε): 323 (4.53), 295(4.5), 239(4.4), 216(4.6) nm
36-16		mp: 125-126°C; IR νmax (cm-1): 3423, 1646, 1593, 1546, 1467, 1360, 1255, 1138, 1020, 970, 857, 817. 1H NMR (CD3COCD3, 500 MHz): δ 2.8 (2H, t, J = 6.8 Hz), 3.5 (2H, q, J = 6.8 Hz), 3.8 (3H, s), 3.8 (3H, s), 6.5 (1H, d, J = 15.6 Hz), 6.7 (1H, dd, J = 8.0, 2.0 Hz), 6.8 (1H, d, J = 8.0 Hz), 6.8 (1H, d, J = 8.0 Hz), 6.8 (1H, d, J = 2.0 Hz), 6.9 (1H, dd, J = 8.0, 2.0 Hz), 7.0 (1H, d, J = 2.0 Hz), 7.2 (-NH, br. s), 7.5 (1H, d, J = 15.6 Hz), 8.2 (-OH, s), 8.4 (-OH, s). EI-MS m/z (%): 343 (M+, 10), 163(100), 151 (16)UV(MeOH) λmax (logε): 322(4.3), 287(4.3), 2.30(4.3), 219(4.5)nm.
36-25B		mp: 140-142°C; IR νmax (cm-1): 3416, 1647, 1600, 1533, 1363, 1280, 1111, 972, 844, 818. 1H NMR (CD3COCD3, 500 MHz): δ 2.8 (2H, t, J = 7.5 Hz), 3.5 (2H, quin, J = 7.5 Hz), 3.8 (3H, S), 6.4 (1H, d, J = 15.6 Hz), 6.8 (1H, d, J = 8.1 Hz), 6.8 (2H, d, J = 8.5 Hz), 6.9 (1H, dd, J = 8.1, 2.0 Hz), 7.1 (H, d, J = 2.0), 7.2 (2H, d, J = 8.5), 7.3 (1H, brs, NH), 7.4 (1H, d, J = 15.6), 8.1 (1H, brs, OH), 8.3 (1H, brs, OH).UV(MeOH) λmax (logε): 322 (4.3), 294 (4.2), 240 (4.2), 222 (4.4)nm.

cells by using lysis buffer, and 10^6 cells were seeded in 24-well plates with 1 mL of RPMI-1640 medium, supplemented with 5% FBS, recombinant murine granulocyte-macrophage colony stimulating factor (GM-CSF; 500 U/mL; PeproTech, Rocky Hill, NJ, USA), 4 mM L-glutamine, 25 mM HEPES (pH 7.2), 50 μM 2-mercaptoethanol, 0.25 μg/mL amphotericin, 100 U/mL penicillin, and 100 μg/mL streptomycin in an incubator containing 5% CO_2 at 37 °C. The medium was replaced every alternate day with fresh medium containing GM-CSF, and the nonadherent cells were transferred to a new plate so as to minimize contamination by macrophages. On day 6 of the culture, the nonadherent cells (BM-DCs) were collected and treated with different chemical compounds for 48 h. This was followed by flow cytometry analyses for examining the expression of surface markers on the BM-DCs prior to their use in other experiments. The $CD11c^+$ cells (indicated as DCs) were gated for analyzing the expression of CD80, CD86, and major histocompatibility complex class II (MHC II). The $CD11c^+$ cells comprised 85% of the total cells in the culture, as depicted in Fig. 2**A**.

Flow cytometry

The BM-DCs (5×10^5) were stained with Alexa Fluor 488-conjugated anti-I-A/I-E [MHC II], phycoerythrin (PE)-conjugated anti-CD86, allophycocyanin-conjugated anti-CD80, or PE-cyanine7-conjugated anti-CD11c (eBioscience, San Diego, CA, USA) at 4 °C for 30 min. The final concentration of the antibodies was 0.2 μg/ml. The cells were washed and suspended in 0.5 mL phosphate-buffered saline along with 0.1% sodium azide and analyzed by flow cytometry (FACSCanto; Becton Dickinson, Mountain View, CA, USA). A total of 2×10^5 cells were counted, and the frequency of each cell-surface marker was determined using FACSDivia and Flow *J* software, provided by Becton Dickinson. The cells in the control setup were suspended only in the medium. The flow cytometer was regularly calibrated with CaliBRITE beads (Becton Dickinson).

Cytotoxicity assay

The cytotoxicity of the compounds was assessed by the 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay according to a previously described protocol [14]. The BM-DCs were cultured in 24-well plates and treated with different concentrations of caffeic amides for 48 h, after which 50 μL of 2 mg/mL MTT (Sigma-Aldrich) was added to each well and incubated for further 3 h. The crystals formed in the wells were dissolved with dimethyl sulfoxide (DMSO), and the optical density was measured at 570 nm.

$CD4^+$ T cell-differentiation assay

On day 6 of the culture, the BM-DCs were treated with different concentrations of 36 M for 10 min and cultured in the presence or absence of OVA (100 μg/mL) combined with TSLP (20 ng/mL) for 48 h. On day 8 of the culture, the BM-DCs were treated with mitomycin C (50 μg/mL) dissolved in DMEM, for 30 min, and washed thrice with HBSS. The BM-DCs were then collected for the cell proliferation assays. Freshly isolated spleen cells from DO11.10 BALB/c transgenic mice, which carried an MHC class II restricted rearranged T cell receptor transgene on an H2d background reactive to OVA peptide antigen, were purified by positive selection using anti-$CD4^+$ microbeads, according to the manufacturer's instructions (Stem Cell Technologies, Vancouver, Canada). The purity of $CD4^+$ T cells were analyzed by flow cytometry ($CD4^+$ T cells: > 90%). The freshly isolated $CD4^+$ T cells (2×10^5 cells) were

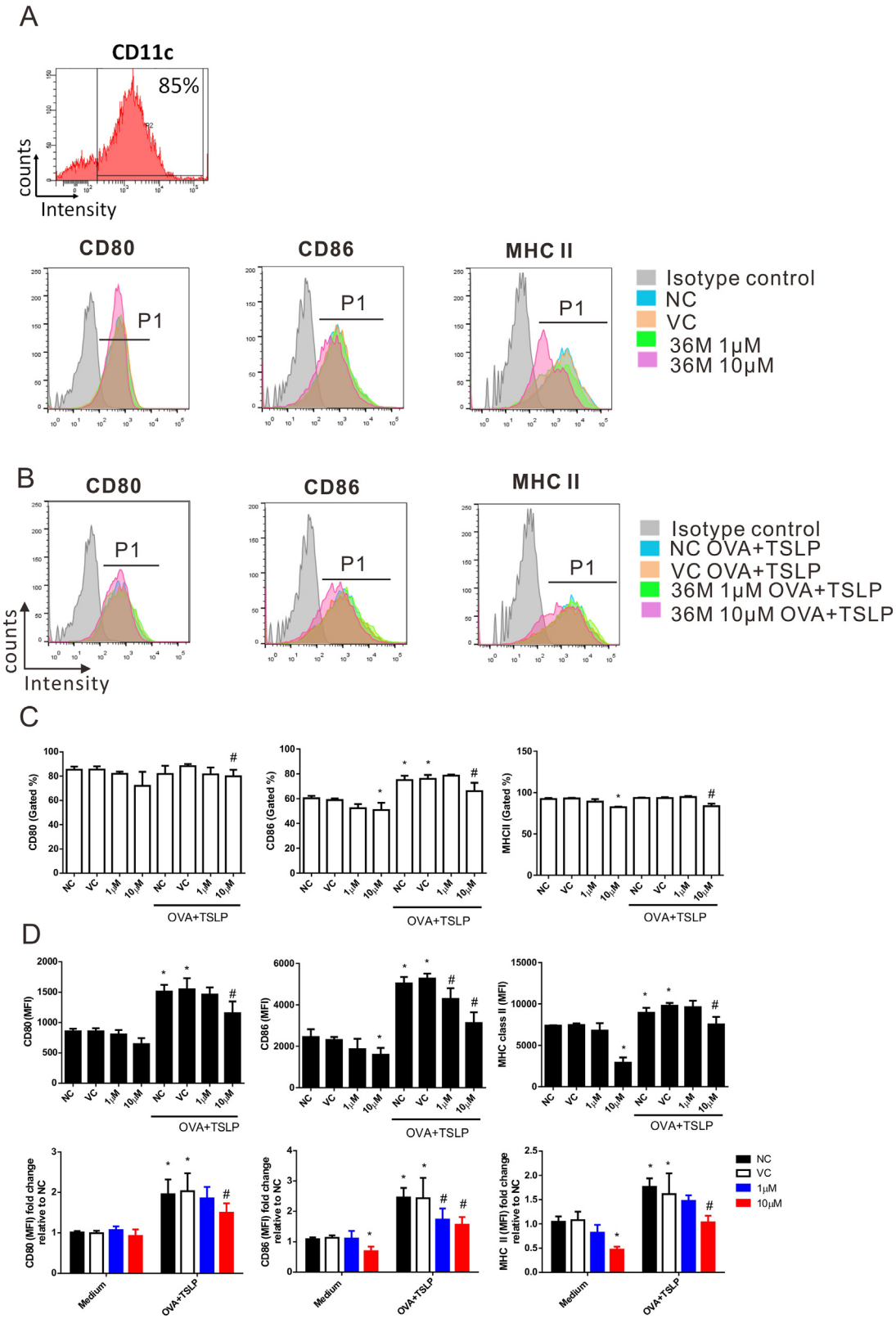


Fig. 2. The caffeic amide, 36 M, inhibits the OVA + TSLP-induced maturation of BM-DCs. Murine BM-DCs (5×10^5 cells) were cultured in 24-well plates and pretreated with 36 M for 10 min, followed by treatment with OVA (100 μg/mL) and TSLP (20 ng/mL) for 48 h, for allowing differentiation into T_H2-polarizing DCs. The expression of surface markers, including CD80, CD86, and MHC II, were detected by flow cytometry. (A) (B) Representative results of flow cytometry, including (C) the gating percentage, and (D) MFI related to the expression of CD80, CD86, and MHC II. The data are expressed as the mean ± SD ($n \geq 3$). *$p < 0.05$, compared to the negative control (NC) or vehicle control (VC; 0.1% DMSO), #$p < 0.05$, compared to the VC- treated with OVA + TSLP group.

co-cultured with the BM-DCs at a ratio of 10:1, in round-bottomed 96-well microtiter plates. After 4 days of culture, the mixed cell cultures were pulsed with 5-bromo-2-deoxyuridine (BrdU) for measuring cell proliferation. Only for the T cell proliferation assay, the $CD4^+$ T cells (2×10^5 cells) were cultured in 36 M for 48 h, and then pulsed with BrdU for analyzing cell proliferation. Briefly, 2 nmol of BrdU was added to each well, and the culture plate was incubated at 37 °C for 8 h. The amount of BrdU incorporated into the T cells was measured using an anti-BrdU monoclonal antibody included in the enzyme-linked immunosorbent assay (ELISA) kit, according to the manufacturer's instructions (Roche Diagnostics GmbH, Mannheim, Germany).

Cytokine assays

The cell culture supernatants were collected after treatment with different drugs, and stored at -20 °C prior to analysis by ELISA, according to the manufacturer's instructions (Roche Diagnostics GmbH). The standards were prepared from recombinant mouse interferon (IFN)-γ and IL-4 (R&D Systems). The detection limits of IL-4 and IFN-γ were 15.6 and 31.2 pg/ml, respectively.

Statistical analysis

All the results are presented as the mean ± standard deviation (SD) of at least three experiments. Group comparisons were performed using two-way analysis of variance, followed by Tukey's multiple comparisons *post hoc* test. A $p < 0.05$ was considered to be significant.

Results

Cytotoxicity of caffeic amides to BM-DCs

The chemical structure and NMR data of the different caffeic amides are provided in Table 1. In order to determine the toxicity of the caffeic amides to the BM-DCs, the BM-DCs were cultured in the presence of each caffeic amide (refer Fig. 1**A** for chemical structures), at concentrations of 0.1, 1, and 10 μM, for 48 h, which revealed that the caffeic amides were not cytotoxic to the BM-DCs (Fig. 1B). The cytotoxic effects of 36 M were further confirmed over different time periods, and as depicted in Fig. 1C, 36 M did not produce significant cytotoxicity in the BM-DCs when incubated for 4, 16, and 24 h. Based on these results, we established the concentration range at which the caffeic amides were to be used for the subsequent experiments.

The caffeic amide, 36 M, inhibits BM-DC maturation

Mature DCs are characterized by high levels of MHC II expression, as well as increased expression of the T cell co-stimulatory molecules, CD86 and CD80. We therefore investigated the effect of caffeic amides on the expression of surface markers in the BM-DCs. As shown in Fig. 2A, the expression of $CD11c^+$ in the DCs reached 85% after 6 days of culture. The expression levels of CD80, CD86, and MHC II were analyzed by gating the $CD11c^+$ DCs. We treated the BM-DCs with OVA, which acted as an antigen, and TSLP, for stimulating the transformation of DCs into T_H2-polarizing DCs. OVA is an allergen, and TSLP is released by the epithelial cells to induce the activation of DCs involved in the differentiation of T_H2 cells [15]. As shown in Table 2, the BM-DCs were treated with 10 μM of each caffeic amide derivative for 48 h. It was observed that only 36 M suppressed the mean fluorescence index (MFI) expression of OVA + TSLP-induced MHC II, CD80, and CD86. The subsequent experiments were therefore performed to evaluate the effect of 36 M on the maturation and activation of BM-DCs.

The caffeic amide, 36 M, inhibits BM-DC-induced allergen-specific differentiation of T_H2 cells

In order to further investigate the effect of 36 M at different dosages on the maturation of BM-DCs, the BM-DCs were treated with 36 M at concentrations of 1 μM and 10 μM. Treatment with 10 μM, but not 1 μM, 36 M for 48 h inhibited the expression of CD80, CD86, and MHC II (Fig. 2). The effects of 36 M on the OVA + TSLP-stimulated maturation of BM-DCs were subsequently evaluated, and the MFI of CD80, CD86, and MHC II were observed to increase, and were subsequently suppressed by treatment with 10 μM 36 M. In order to evaluate the effects of 36 M on the differentiation of T_H2 cells driven by the OVA + TSLP-stimulated activation of the BM-DCs, OVA-specific $CD4^+$ T cells were isolated from the spleen of DO11.10 mice and co-cultured with the BM-DCs for 48 h for analyzing the proliferation and differentiation of $CD4^+$ T

Table 2
Surface markers expression on BM-DCs after treatment of caffeic amide derivatives.

	CD80 %	CD80 MFI	CD86%	CD86 MFI	MHC II %	MHC II MFI
NC	85.3 ± 2.6	857.7 ± 39.7	60.3 ± 2.1	2446 ± 379.2	92.3 ± 1.1	7383 ± 50.1
VC	85.5 ± 2.7	856.8 ± 50.2	58.9 ± 1.2	2304 ± 148.2	92.9 ± 0.7	7472 ± 195.2
36	86.2 ± 1.2	800.3 ± 82.9	57.5 ± 2.2	2358 ± 168.4	94.9 ± 5.4	7632 ± 330.7
36H	84.5 ± 3.0	874 ± 22.5	55.9 ± 2.6	2201 ± 125.7	89.1 ± 2.1	7577 ± 528.2
36 M	72.1 ± 11.5	646 ± 96.8	52.7 ± 4.7*	1595 ± 328.9*	82.3 ± 0.8*	2916 ± 626.3*
36-6	76.5 ± 4.2	810.3 ± 61.9	54.2 ± 2.5	2134 ± 155.6	93.2 ± 4.3	7656 ± 518.2
36-16	82.1 ± 7.9	853.3 ± 52.2	58.1 ± 3.8	2359 ± 122.7	93.4 ± 6.9	7656 ± 518.2
36-25B	80.0 ± 3.3	885.3 ± 72.2	62.6 ± 1.7	2330 ± 172.9	89.4 ± 2.4	7632 ± 600.6
	OVA + TSLP					
	CD80 %	CD80 MFI	CD86%	CD86 MFI	MHC II %	MHC II MFI
NC	81.9 ± 6.7	1509 ± 111.6*	75 ± 3.4*	5039 ± 309.5*	93.4 ± 0.7	8947 ± 295.8*
VC	88.3 ± 1.7	1550 ± 180.9*	76 ± 3.1*	5274 ± 240*	93.3 ± 1.2	9774 ± 342*
36	87.7 ± 1.5	1545 ± 135.7	86.2 ± 1.2	5215 ± 91.2	94.1 ± 4.9	9989 ± 245.4
36H	88.6 ± 3.0	1643 ± 49.9	84.5 ± 3.0	5186 ± 330.3	90.6 ± 7.1	9832 ± 394.1
36 M	79.9 ± 5.3#	1155 ± 192.2#	66.1 ± 6.7#	3126 ± 212.7#	83.6 ± 3.1#	7528 ± 929#
36-6	89.5 ± 1.8	1543 ± 162.4	76.5 ± 4.2	5006 ± 445.5	95.4 ± 3.8	9866 ± 1362
36-16	89.3 ± 1.1	1655 ± 47.6	82.1 ± 7.9	5274 ± 483.4	96.4 ± 5.3	10394 ± 969.8
36-25B	88.9 ± 1.3	1638 ± 46.7	80.0 ± 3.3	5272 ± 240.4	93.8 ± 3.7	9874 ± 247.7

Murine Bone marrow derived dendritic cells were treated with different caffeic amides for 48 h and then cells surface markers were detected by flow cytometry. Data were expressed by Mean ± SD #$p < 0.05$ *vs.* vehicle control (VC) group. *$p < 0.05$ *vs.* OVA + TSLP treated VC group.

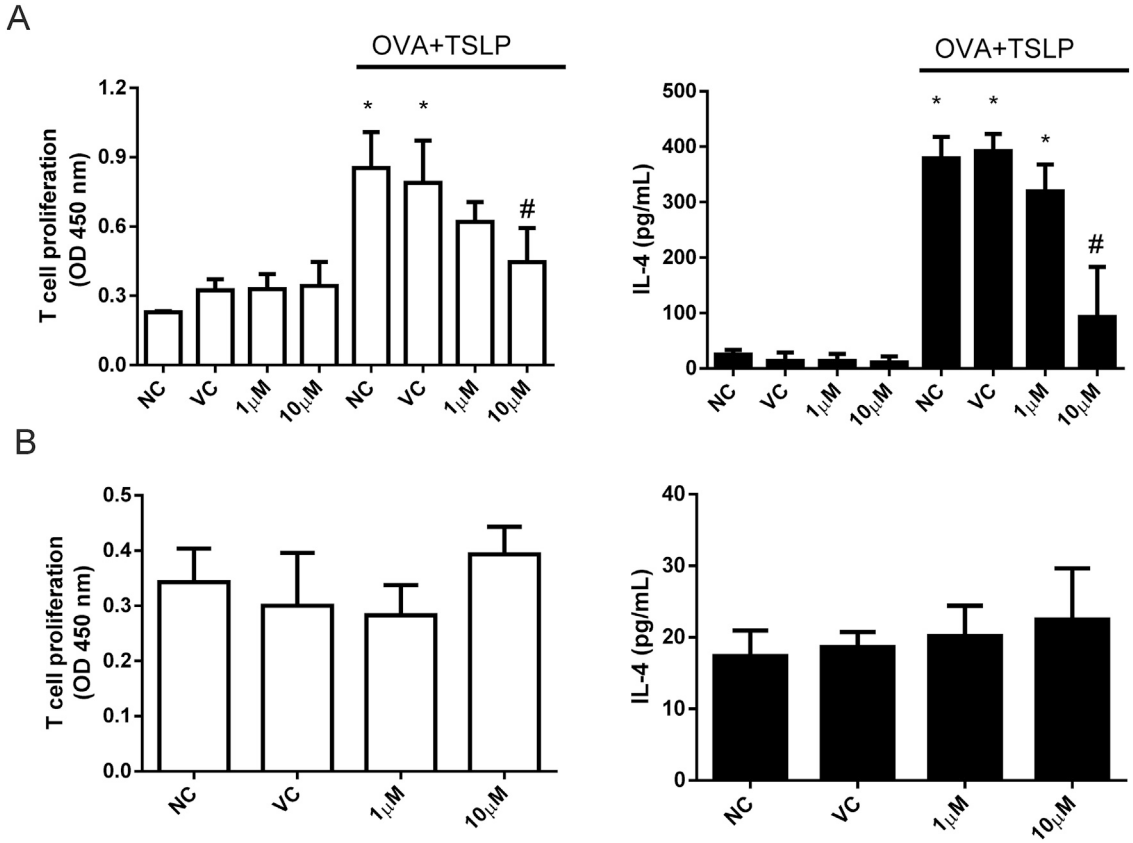


Fig. 3. The caffeic amide, 36 M, suppresses the differentiation of T_H2 cells driven by OVA + TSLP-induced BM-DCs. (A) Murine BM-DCs (5×10^5 cells) were cultured in 24-well plates and pretreated with 36 M for 10 min, followed by treatment with OVA (100 μg/mL) + TSLP (20 ng/mL) for 48 h. Freshly isolated $CD4^+$ T cells (2×10^5 cells) from the spleen of DO11.10 mice were co-cultured with 2×10^4 mitomycin C-treated BM-DCs in the presence of various treatments in 96-well plates for 4 days. (B) $CD4^+$ T cells (2×10^5 cells) freshly isolated from the spleen of DO11.10 mice were treated with different doses of 36 M for 48 h. Cell proliferation was analyzed by the incorporation of BrdU and the medium was analyzed for measuring the production of cytokines, as described in the methods section. The data are expressed as the mean ± SD ($n \geq 3$). *$p < 0.05$, compared to the negative control (NC) or vehicle control (VC; 0.1% DMSO). #$p < 0.05$, compared to the VC- treated with OVA + TSLP group.

cells (Fig. 3). After 48 h, the OVA + TSLP-stimulated DCs markedly induced the proliferation and differentiation of $CD4^+$ T cells by inducing the release of IL-4 (Fig. 3A). It was observed that treatment with 10 μM 36 M significantly inhibited the proliferation of $CD4^+$ T cells and production of IL-4, which had been induced by the OVA + TSLP-activated BM-DCs (Fig. 3A). Moreover, IFN-γ production was not observed following treatment with 36 M (data not shown), which indicated therefore, that 36 M did not inhibit the differentiation of T_H2 cells *via* T_H1 cell activation. Without co-treatment with DCs, the marked increase in CD4 + T cell activation was absent in the groups treated with 36 M Fig. 3B).

Discussion

DCs are critical initiators of allergic reactions, and therefore represent an important therapeutic target for allergic diseases, such as asthma [11], atopic dermatitis [16], and food allergies [17]. In this study, we observed that treatment with the caffeic amide derivative 36 M, suppressed the maturation of BM-DCs by inhibiting the expression of CD80, CD86, and MHC II, and by suppressing the differentiation of T_H2 cells. To the best of our knowledge, this study is the first to investigate the effects of a caffeic amide derivative on the maturation of DCs, and to evaluate its potential for the treatment of allergic diseases.

Among the known caffeic amide derivatives, caffeic acid phenethyl ester (CAPE), extracted from propolis, has been previously studied for its antitumor [18], antioxidant [19], and anti-inflammatory [20] properties. Since the use of esters is restricted by their low metabolic stability, most of the studies modified CAPE to caffeic acid phenethyl amide (CAPA), referred to as "36″ in this study, to increase its *in vivo* stability [21], since amides possess a high hydrolytic activation energy and are capable of escaping hydrolysis by the plasma esterases. CAPA also exhibits antioxidant [22] and anti-inflammatory [23,24] properties, and can also improve glucose hemostasis and the symptoms associated with diabetes mellitus [25]. However, surprisingly, 36 (CAPA) failed to exhibit the anticipated effects in the maturation of BM-DCs (Table 2), with only 36 M showing significant inhibition of BM—DC maturation and T_H2 cell differentiation. Analysis of the structure-activity relationship of the six caffeic amide derivatives might explain why only 36 M exhibited inhibitory effects on the OVA + TSLP-induced maturation of BM-DCs. Among the six caffeic amide derivatives considered in this study, only the side chain of R in 36 M is an alkyl group (octyl group), whereas the side chains of the other compounds are phenyl groups. Since the electron density in alkyl groups is low, they are considered to be non-polar substituents; however, the electron density in phenyl groups is higher, which makes them more polar than alkyl groups. The lipid bilayer in the cell membrane is non-polar in nature, and has a high affinity for non-polar groups. Owing to the non-polar nature of its side chain, 36 M had a higher ability to pass through the cell membrane than the other compounds in this study, and showed inhibitory effects on the maturation of DCs.

Tsai and coworkers [22] previously reported that 36 M possesses antioxidant properties and protective effects against oxidative stress-induced HepG2 cell toxicity, by ameliorating

mitochondrial dysfunction. In this study, we evaluated the effect of short-term (10 min and 6 h) and long-term (48 h) OVA + TSLP treatments on oxidative stress in BM-DCs. However, it was observed that OVA + TSLP treatments did not induce the production of H_2O_2 (Supporting Information, Fig. S1). Moreover, no marked production of H_2O_2 was observed in the groups treated with 1 μM and 10 μM 36 M, as well as in the groups treated with 36 M combined with OVA + TSLP. Therefore, the inhibition of DC maturation by 36 M was not associated with the suppression of ROS production. The mechanisms associated with the inhibition of BM—DC maturation by 36 M require further investigation.

Since DCs are the key antigen-presenting cells in inducing the activation and differentiation of $CD4^+$ T cells, several diseases related to immune dysfunction, including autoimmune disorders, allergic diseases, cancer-related diseases, and inflammatory disorders, can potentially benefit by targeting DCs, which would alter the function or activation of T_H cells, and promote the long-term alleviation of inflammatory dysfunction. Additionally, there are numerous strategies for evaluating the therapeutic efficacy of drugs targeting allergic diseases by using DCs as a cell-based platform. First, the evaluation of DC-induced activation of T_H1 cells provides insights into the pathways capable of inhibiting T_H2 cell activation [26,27]. Second, the evaluation of DC-induced activation of regulatory T (Treg) cells can provide insights into the Treg-cell-related inhibition of T_H2 cell activation [28]. Third, the activation of T_H2 cells can be directly evaluated by the inhibition of DC maturation [29]. The third strategy was first developed by us and can be effectively utilized for *in vitro* and *in vivo* studies for potentially reducing the side effects associated with the induction of T_H1 cells or the activation of Treg cells. The third strategy was employed in this study for investigating the therapeutic potential of caffeic amides against allergic diseases. The study further determined whether 36 M could attenuate the expression of surface markers in BM-DCs and suppress the activation of T_H2 cells without increasing the expression of surface markers on DCs for driving the differentiation of T_H1 cells. The results of this study indicated that 36 M directly targeted the OVA + TSLP-induced T_H2 cell activation pathway.

In conclusion, we evaluated the effects of six caffeic amides on the maturation of DCs, which revealed that 36 M (*N*-octyl caffeamide) suppressed DC maturation and T_H2 cell activation, therefore suggesting its potential in the treatment of allergic diseases.

Conflict of interest

The authors announce no conflict of interest or competing financial interest.

Acknowledgement

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.pharep.2018.10.014.

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Supplementary material

Methods

Detection of reactive oxygen species (ROS) production

2×10^4 BM-DCs were culture in 96-well microtiter plates. For 10 min and 6h ROS production, cells were stained with the cell permeant reagent 2',7' –dichlorofluorescin diacetate (DCFH-DA) 20μM for 30min, washed twice with PBS, and treated with different doses of 36M for indicated time periods. For 48h ROS production, cells were treated with 36M for 48h, stained with DCFH-DA for 30mins, and then washed PBS twice. ROS production was measured fluorescence (Ex/Em=485/535 nm) in a fluorescence microplate reader (Thermo).

FIGURE LEGENDS

Figure S1. **Effect of 36M on ROS production in BM-DC cells.** For 6h and 48h ROS production, BM-DCs were pretreated 36M for 10min, followed by treatment with OVA (100 μg/mL) + TSLP (20 ng/mL) for 48 h. For 10min ROS production, BM-DCs were co-treated with 36M and OVA+TSLP for 15min. Cells were stained with DCFH-DA 20μM for 30min as described in supporting information and ROS production was measured fluorescence (Ex/Em=485/535 nm) in a fluorescence microplate reader. Cells were treated with 62.5μM H_2O_2 for 15 min as a positive control.

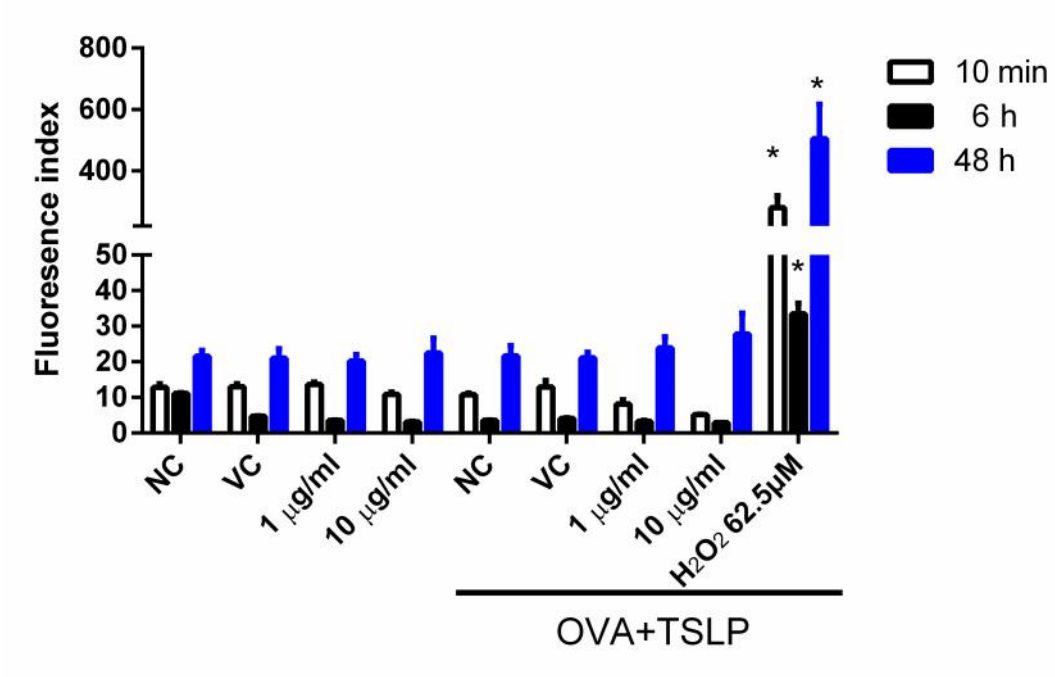
Fluoresence index
800
600
400
50
40
30
20
10
0
10 min
6 h
48 h
*
*
*
NC
VC
1 μg/ml
10 μg/ml
NC
VC
1 μg/ml
10 μg/ml
H2O2 62.5μM
OVA+TSLP

Figure. S1