Abstract.
Dendritic cells (DCs) are crucial regulators of allergic diseases. Hesperetin, an important bioactive compound in Chinese traditional medicine, has antioxidant and anti-allergic properties. In this study, we examined whether hesperetin influences surface molecule expression, cytokine production, the capacity to induce T cell proliferation, and the underlying signaling pathway in monocyte-derived DCs from patients with allergic asthma. The results show that hesperetin significantly suppressed Der p 1-induced HLA-DR, CD86 and CD83 expression in DCs. However, the secretion of IL-10 was not affected. Hesperetin-treated DCs exhibited a reduced ability to stimulate autologous CD4+ T cells, accompanied by less Th2 polarization. In addition, the Der p 1-induced phosphorylation of IκBα and the translocation of NF-κB p65 were inhibited in the presence of hesperetin. These novel findings provide insight into the immunopharmacological role of hesperetin in DC-based allergic diseases.

Introduction
Asthma is an allergic disorder characterized by Th2-mediated eosinophilic airway inflammation, airway hyperresponsiveness and airway remodeling (1). Dendritic cells (DCs) are professional antigen-presenting cells that have the ability to stimulate naive T cells and to initiate the differentiation of CD4+ T cells into Th1 or Th2 subsets (2). There are reports which suggest that in vivo DCs play a crucial role in the establishment of the allergic inflammatory reaction: their numbers increase in the lungs of asthmatic patients (3); bronchial epithelial DCs issued from asthmatic patients activate CD4+ T cells with a preferential secretion of Th2 cytokines such as IL-4 and IL-5 (4); selective DC depletion in ovalbumin-sensitized mice leads to a decrease in immunoglobulin (Ig)E production, the abolition of Th2 response and a lack of eosinophil recruitment (5). DCs are therefore becoming a viable drug target in allergic asthma due to their crucial role in the initiation of immune response. Steroids and immunosuppressants have been used to treat allergic diseases (6), but have failed to effect significant improvements. Traditional herbal medicines have been developed for allergic diseases, and have received increasing attention due to their effectiveness (7).

Hesperetin (5,7,3′-trihydroxyl-4′-methoxyl-flavanone) is a bioactive compound that belongs to a class of flavonoids called flavanones, which are abundant in citrus fruits. The compound is used in Chinese traditional medicine and is known to possess several biological and pharmacological activities, including antioxidant (8), anti-inflammatory (9), and anticarcinogenic properties (10). It is also specifically known to alter signal transduction pathways (11). Recently, hesperetin was reported to exhibit an anti-allergic action by inhibiting histamine release from RBL-2H3 cells (12,13). However, the cellular targets of hesperetin in the immune system have yet to be studied.

In this study, we aimed to investigate the effect of a non-cytotoxic concentration of hesperetin on the maturation and function of monocyte-derived DCs from patients with allergic asthma. Our results indicate that hesperetin is a potent inhibitor of DCs. These findings provide new insight into the immunopharmacology of hesperetin.


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Materials and methods
Reagents. Hesperetin was obtained from Sigma Chemical Co. (St. Louis, MO, USA) and dissolved in DMSO (0.1%). The recombinant human (rh) granulocyte macrophage-colony-stimulating factor (GM-CSF) rhIL-4 was purchased from Peprotech (London, UK).

Subjects. Ten non-smoking patients with allergic asthma (6 females and 4 males) were recruited from the database of the China Asthma Alliance Jiangsu Branch according to the Global Initiative for Asthma (GINA) 2006 guidelines (14). All allergic patients presented positive skin prick tests for the
house dust mite *Dermatophagoides pteronyssinus*, as well as elevated total serum IgE concentrations (206.6±30.8 IU/ml).

None of the subjects had respiratory infections and none had used corticosteroids in the previous 4 weeks. Short-acting β-agonists alone were inhaled on demand as rescue medication, but were discontinued 12 h prior to blood sampling. All subjects gave their written informed consent. The study was approved by the Jiangsu Province Hospital Ethics Committee.

**Generation of monocyte-derived dendritic cells.** DCs were generated from lymphocyte-depleted peripheral blood mononuclear cells (PBMCs) according to established procedure (15). Briefly, PBMCs were separated on Ficoll (Haoyang Biotech, Tianjing, China) from samples of heparinized venous blood obtained from patients. Monocytes were derived from PBMCs depleted of NK, B and T cells with anti-CD16, anti-CD19 and anti-CD3, as well as with goat anti-mouse Ig-conjugated magnetic beads (Miltenyi Biotech, USA). The cells were then cultured (2x10⁶ cells/well) in 6-well flat-bottomed culture plates (Coster, Cambridge, MA, USA) in RPMI-1640 supplemented with 1% antibiotics and 10% fetal calf serum (Gibco, Grand Island, NY) for 7 days in the presence of 1000 U/ml GM-CSF and 500 U/ml IL-4. Every other day, half of the medium in the cell cultures was replaced with fresh medium.

**Stimulation of dendritic cells.** On day 7, immature DCs (iDCs) were harvested. To explore the effects of hesperetin on DC maturation, iDCs (1x10⁶ cells/ml) were pulsed with medium alone or with predetermined non-cytotoxic doses of house dust mite allergen (100 ng/ml Der p 1) (ALK-Scherax, Hamburg, Germany) in the presence or absence of hesperetin (12.5, 25 and 50 μM). Supernatants were collected after a 24-h culture. After extensive washing, cells were coincubated with T cells or subjected to other analyses.

**MTT assay.** Cell viability was determined using the MTT assay in 96-well plates as previously described (16). The cells (2x10⁶ cells/well) were treated with various concentrations of hesperetin for 24 h, then incubated with MTT (Sigma Chemical Co.) for 4 h at 37°C. After the removal of unconverted MTT, 100 μl of isopropanol (in 0.04 N hydrochloric acid) was added to dissolve the formazan crystals. Absorbance was read at 570 nm using the CliniBio-128C spectrophotometer (Austria). Cell viability was calculated as the relative absorbance compared to the control.

**Flow cytometry.** On day 8, the DCs were collected, washed and incubated for 30 min at 4°C with the following fluorescent-labeled mouse anti-human monoclonal antibodies (mAbs): fluorescein isothiocyanate (FITC)-conjugated anti-CD80, anti-CD83 or anti-CD1a; Phycoerythrin (PE)-conjugated anti-CD86, anti-HLA-DR or anti-CD14 (eBioscience, San Diego, CA). After washing, the cells were fixed in 1% paraformaldehyde and analyzed using a FACSCalibur (Becton-Dickinson).

**Proliferation assay.** Autologous T cells were isolated and enriched using RossetSep CD4 reagent (StemCell Technologies) as previously described (17). DCs and T cells were used as stimulators and responders, respectively. Stimulators were co-cultured with responders (2x10⁴ cells/well) in triplicate at serial ratios (1:100, 1:50, 1:20 and 1:10) in 96-well plates (200 μl/well) in 5% CO₂ at 37°C for 96 h, then pulsed with 0.5 μCi/well of [³H] thymidine for the final 16 h before liquid scintilligraphy. The counts per min (cpm) of triplicate wells were calculated.

**Dendritic cell-T-cell cocultures.** Various treated DCs were cocultured with autologous CD4⁺ T cells at a ratio of 1:10 for 48 h. The supernatants were harvested and assayed for IL-4 and IFN-γ production.

**Cytokine assay.** Cellular supernatants were harvested on day 8 to measure the levels of IL-10 and IL-12 p70 by enzyme-linked immunosorbent assay (ELISA, Biosource, USA). IL-4 and IFN-γ by T cells cocultured with DCs was measured in the supernatants by ELISA according to the manufacturer’s protocols.

**Western blot analysis.** On day 7, iDCs were recovered and pretreated with 50 μM hesperetin for 40 min, and then exposed to 100 ng/ml Der p 1 for 60 min at 37°C. Cells were washed twice in ice-cold PBS, and cytoplasmic and nuclear proteins were extracted (15). Denatured proteins (80 μg) were separated on 10-12% SDS-PAGE and blotted onto polyvinylidene difluoride membranes. The membranes were probed with either rabbit anti-human p65, mouse anti-human IκBα, mouse anti-human phospho-IκBα (pIkBα, Ser32/36) or mouse anti-human β-actin antibody (Cell Signaling Technology, Beverly, MA; all at a 1:1000 dilution) overnight at 4°C, and then incubated with a horseradish peroxidase-conjugated secondary antibody (Cell Signaling Technology). The immunoblots were visualized with an enhanced chemiluminescence detection system (Pierce, Rockford, IL).

**Statistics.** Data are expressed as the means ± SEM of the indicated number of experiments. Statistical significance was estimated using the Student’s t-test for unpaired observations, and one-way analysis of variance for the comparison of differences. P<0.05 was considered significant.
Results

Hesperetin inhibits the phenotypic maturation of monocyte-derived dendritic cells. After 7 days of culture with GM-CSF and IL-4, monocytes differentiated into large round and non-adherent cells exhibiting protruding veils or dendrites typical of DCs. Generated cells were analyzed for the expression of the monocyte marker CD14 and the DC markers CD1a, HLA-DR, CD80, CD86 and CD83. The results show that these cells decreased the expression of CD14 and elevated the expression of CD1a, whereas HLA-DR, CD80, CD86 and CD83 were expressed at low levels (data not shown). This profile is specific for iDCs.

In some experiments, we attempted to determine whether hesperetin influences the phenotypic maturation of DCs. We first evaluated the effect of hesperetin on the viability of DCs using the MTT assay. As depicted in Fig. 1, even at 50 μM hesperetin had little cytotoxicity when incubated with cells for 24 h. Therefore, all further experiments were performed using 50 μM hesperetin.

As shown in Table I, pulsing with Der p 1 induced the increased expression of CD86, HLA-DR and CD83 (all P<0.05) but not CD80 (P>0.05) in iDCs. Treatment with hesperetin significantly inhibited the Der p 1-induced expression of CD86, HLA-DR and CD83 (all P<0.05) in DCs.

Hesperetin does not influence IL-10 production in Der p 1-stimulated dendritic cells but impairs their ability to induce T cell proliferation. As IL-12 and -10 are known to play key roles in the modulation of T cell response, their production by DCs was evaluated. When iDCs from asthmatic patients sensitive to the house dust mite were incubated with Der p 1, a significant production of IL-10 were observed (Fig. 2A), whereas IL-12 was not detected in any of the culture supernatants from the DCs tested (data not shown). Notably, hesperetin did not impair the production of IL-10 (Fig. 2A).

To test whether hesperetin inhibits the accessory function of Der p 1-stimulated DCs on T cell proliferation, DCs from 8-day cultures that had been incubated with or without hesperetin for 24 h were tested for their capacity to stimulate autologous T cells. As expected, iDCs markedly promoted T cell proliferation in response to Der p 1 (P<0.05 for all DC/T cell ratios, with the exception of the DC/T cell ratio of 1:100). However, hesperetin impaired the capacity of Der p 1-pulsed DCs to stimulate T cell proliferation (P<0.05 for all DC/T cell ratios, with the exception of the DC/T cell ratio of 1:100) (Fig. 2B).

Hesperetin inhibits the capacity of Der p 1-pulsed dendritic cells to induce Th2 responses. To further clarify the effect of

Table I. Expression of CD80, CD86, CD83 and HLA-DR on dendritic cells.

<table>
<thead>
<tr>
<th>CD80 (%)</th>
<th>CD86 (%)</th>
<th>CD83 (%)</th>
<th>HLA-DR (%)</th>
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<tbody>
<tr>
<td>iDCs</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Medium</td>
<td>25.69±4.30</td>
<td>37.18±3.96</td>
<td>10.99±2.70</td>
</tr>
<tr>
<td>Der p 1</td>
<td>42.82±6.85</td>
<td>63.61±6.71a</td>
<td>45.18±2.87a</td>
</tr>
<tr>
<td>Der p 1 + H</td>
<td>38.00±3.42</td>
<td>41.28±4.53b</td>
<td>24.95±5.64b</td>
</tr>
</tbody>
</table>

iDCs, immature dendritic cells; H, 50 μM hesperetin. Data are presented as the means ± SEM. *p<0.05 vs. iDCs + medium; **p<0.05 vs. iDCs +Der p 1.
Hesperetin-treated DCs on Th1/Th2 balance, we measured IL-4 and IFN-γ in the supernatants of the cultures. Naive autologous T cells primed with Der p 1-pulsed DCs differentiated into Th2 lymphocytes because they secreted pronounced amounts of IL-4 and low levels of IFN-γ compared to samples of non-stimulated DCs (Fig. 3A and B). In contrast, T lymphocytes primed with DCs matured in the presence of hesperetin displayed a reduced production of IL-4.

Hesperetin suppresses the Der p 1-induced nuclear translocation of NF-κB p65 in dendritic cells. Normally, NF-κB is present in the cytoplasm in an inactive state and bound to a member of the IκB inhibitor protein family. Following stimulation, IκBα is phosphorylated and degraded. Subsequently, unbound NF-κB translocates to the nucleus and transactivates various downstream genes. Since the activation of NF-κB is an important event in DC maturation (18), and Der p 1 has been shown to promote the nuclear translocation of NF-κB p65 in asthmatic bronchial epithelial cells (19), we explored whether hesperetin affects the Der p 1-induced activation of NF-κB in DCs. Hesperetin pretreated DCs were exposed to 100 ng/ml Der p 1 for 60 min. The results revealed that the increased phosphorylation of IκBα with the nuclear translocation of NF-κBp65 in Der p 1-pulsed DCs was blocked by hesperetin (Fig. 4). These data indicate that hesperetin might inhibit the translocation of NF-κB by disturbing IκBα degradation.

Discussion

During the initiation and maintenance of an immune response (2). Our experimental system used DCs and autologous lymphocytes from patients with allergic asthma, in which allergen stimulation induces a Th2 response (20). Hesperetin is known to possess anti-inflammatory (9) and anti-allergic activity (12,13). The present study evaluated the effects of hesperetin on the expression of surface molecules, cytokine production and T-cell differentiation, as well as the signaling pathway underlying these phenomena in monocyte-derived DCs. The maturation and function of DCs was not inhibited by reduced DC viability, because hesperetin (50 μM) was not toxic to DCs.

By controlling the strength and duration of TCR triggering, the pattern of costimulatory molecule expression and the production of polarizing cytokines, DCs determine the outcome of the primary T cell response of tolerance, unpolarized Th0, or polarized Th1 or Th2 (21). In this study, we demonstrated that treatment with hesperetin inhibited the Der p 1-induced expression of HLA-DR, CD86 and CD83 in DCs. These results suggest that hesperetin may inhibit the phenotypic maturation of DCs. A significant increase in IL-10 but not IL-12 production was also observed in DCs matured in response to Der p 1. However, hesperetin did not impair the production of IL-10. Overall, hesperetin has a major influence on the signals involved in T cell activation.

Changes in surface marker expression and cytokine production were also reflected at a functional level when the allo-stimulatory capacity of DCs in a mixed lymphocyte reaction was analyzed, and naive autologous T cells primed with Der p 1-pulsed DCs from allergic asthma patients differentiated into Th2 lymphocytes. This last is in line with a report by Hammad et al (20). Hesperetin potently inhibited the capacity of mature DCs to stimulate the proliferation of CD4+ T cells, accompanied by a reduction in the production of IL-4. These data indicated that hesperetin inhibited the Th2 polarization induced...
by Der p 1-pulsed DCs, and that this inhibition might depend on the down-regulation of HLA-DR, CD86 expression in DCs. As NF-κB activation controls both the expression of costimulatory molecules and the major histocompatibility complex in DCs (22), we conducted an evaluation of NF-κB activation to characterize the mechanism underlying the ability of hesperetin to inhibit DCs maturation. Der p 1-pulsed DCs clearly exhibited the increased degradation of IkBα and the nuclear translocation of NF-κB p65. However, hesperetin virtually reduced the nuclear translocation of NF-κB p65 by blocking IkBα degradation (23).

IL-12, a DC-derived cytokine, has multiple immunoregulatory functions that activate the Th1 subset. The Th2 cytokine IL-10 plays an essential role in immune tolerance to allergens. A notable observation in this study was that hesperetin did not affect IL-10 levels in DCs. This may have to do with the fact that IL-10 is regulated by STAT3, and not by NF-κB (17,24). We have not been able to evaluate the effect of hesperetin on IL-12 production by mDCs, as IL-12 was not detected in any of the culture supernatants. Previous evidence suggests that NF-κB controls cytokine production, such as that of IL-12, by means of DCs (22). We propose that hesperetin might also regulate IL-12 production. However, future studies examining this hypothesis are required.

In conclusion, NF-κB is a critical transcription factor involved in the pathogenesis of many disorders, including inflammatory diseases such as asthma. Inhibition of NF-κB accordingly results in a reduced capacity of DCs to secrete pro-inflammatory cytokines and to stimulate naive CD4+ T cells. Thus, developing and identifying specific components that modulate NF-κB without adverse side-effects would be beneficial. The current study shows that hesperetin inhibits the maturation and function of DCs from patients with allergic asthma by suppressing the activation of NF-κB. This implies that hesperetin is a candidate therapeutic agent for allergies and inflammation.

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References