
Inhibition effects of *Vitex rotundifolia* on inflammatory gene expression in A549 human epithelial cells

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Background: *Vitex rotundifolia* has long been used in traditional medicine to treat asthma and other allergic diseases.

Objective: To evaluate the anti-inflammatory mechanisms of *V rotundifolia* in cultured A549 human alveolar epithelial cells.

Methods: In the present study, A549 cells were stimulated with tumor necrosis factor α , interleukin 4, and interleukin 1 β to induce expression of chemokines and adhesion molecules involved in eosinophil chemotaxis. The anti-inflammatory effects of *V rotundifolia* on stimulated A549 cells were then evaluated by analyzing eotaxin secretion and eosinophil migration. In addition, the effects of *V rotundifolia* on gene expression profiles in stimulated A549 cells were evaluated by oligonucleotide microarray and real-time reverse transcription–polymerase chain reaction (RTRP).

Results: The *V rotundifolia*-treated A549 cells had significantly suppressed eotaxin secretion and eosinophil migration in a dose-dependent manner. In addition, the results of the microarray analysis and RTRP revealed that inflammation-related genes and cell adhesion-related genes were down-regulated in *V rotundifolia*-treated A549 cells. Furthermore, several genes related to the mitogen-activated protein kinase pathway were down-regulated in *V rotundifolia*-treated A549 cells.

Conclusions: The mechanism responsible for the effects of *V rotundifolia* on A549 cells is closely associated with regulation of the mitogen-activated protein kinase pathway. Thus, *V rotundifolia* may be useful in the treatment of asthma and other allergic diseases.

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INTRODUCTION

Respiratory epithelium plays a key role in airway inflammatory diseases, including asthma, by production of numerous cytokines, chemokines, inflammatory enzymes, and adhesion molecules.^{1,2} The effects of the epithelium are particularly evident in asthma, which is orchestrated by T_H2 cells. T_H2 cells are defined by their secretion of a panel of cytokines, including interleukin (IL) 4 and IL-13, which are known to stimulate the expression of several chemokines, such as eotaxin, monocyte chemoattractant protein (MCP) 4, thymus and activation regulated chemokine, and vascular cell adhesion molecule (VCAM) 1. These chemokines and adhesion molecules play a pivotal role in the infiltration of inflammatory cells into the epithelium. T_H2 cells also produce IL-5, which activates eosinophils in the asthmatic airway.^{3–6} In addition, eosinophils express several membrane receptors, including those for immunoglobulins; cytokines; chemo-

kines, such as eotaxin and regulated upon activation normal T-cell expressed and secreted (RANTES); and adhesion molecules, such as intercellular adhesion molecule (ICAM) 1 and VCAM-1. ICAM-1 is expressed on endothelial and epithelial cells, whereas VCAM-1 is expressed on vascular endothelium.⁷

Vitex rotundifolia is a medical plant that is widely used in China and Korea for the treatment of asthma, pain, cold, headache, migraine, sore eyes, chronic bronchitis, myalgia, and gastrointestinal infections such as bacterial dysentery and diarrhea.^{8,9} Shin et al¹⁰ found that *V rotundifolia* inhibited the allergic reactions of rat peritoneal mast cells by anti-dinitrophenyl IgE. Therefore, this study was conducted to evaluate the anti-inflammatory mechanisms of *V rotundifolia* in A549 cells.

METHODS

Cell Culture

A549 cells (human type 2–like epithelial carcinoma cell line) and EoL-1 cells (human, blood, and leukemia eosinophilic cell line) were obtained from the KCLB (Cancer Research Institute, Seoul, Korea) and Japan RIKEN (<http://www.brc.riken.go.jp>) cell bank, respectively. These cells were then grown and maintained in Roswell Park Memorial Institute (RPMI) medium supplemented with 10% fetal bovine serum (HyClone, Logan, Utah) and penicillin-streptomycin (Invitrogen Life Technologies, Rockville, Maryland) at a concentra-

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tion of 1×10^6 cells per milliliter in 100% humidity at 37°C and 5% carbon dioxide. A549 cells were then plated onto 12-well, flat-bottom, tissue culture plates at a density of 5×10^5 cells per well in hormonally defined RPMI media, as previously described. The medium was changed every 2 days until the cells became 80% to 90% confluent, at which point they were used for the experiments.

Preparation of *V rotundifolia*

Vitex rotundifolia was purchased from the Sun Ten Pharmaceutical Co Ltd (Taipei, Taiwan), powdered to 0.1 g, and then extracted by stirring in 10 mL of distilled water overnight at room temperature using a stirrer. The sample was then centrifuged for 10 minutes at 3,000 rpm, after which the supernatant was removed and sterilized by passing it through a 0.22- μ m syringe filter. The sterilized supernatant was then used for the experiments.

Cytokine Assays

A549 cells were plated at a density of 5×10^5 cells per well in 1 mL of RPMI medium for 24 hours at 37°C. Next, 100-ng/mL tumor necrosis factor (TNF) α (Biosource, Camarillo, California) and 100-ng/mL IL-4 (Biosource) were added, and the cells were then incubated at 37°C for 3 hours to stimulate eotaxin secretion. The cells were then stimulated by treatment with 100-ng/mL TNF- α and 100-ng/mL IL-1 β (Biosource) (T4B treatment). After 24 hours of incubation at 37°C, the A549 cells were washed twice with phosphate-buffered saline and then treated with *V rotundifolia* extract (0, 0.1, 1, or 10 μ g/mL). The cells were then incubated for an additional 48 hours at 37°C. Next, the supernatants were analyzed for eotaxin using an enzyme-linked immunosorbent assay kit (BD Bioscience, San Jose, California). Briefly, flat-bottom 96-well plates were coated with eotaxin-specific monoclonal antibody and then incubated overnight at 4°C. The primary monoclonal antibodies were then discarded, after which the plates were blocked with assay diluent (Pharmingen, San Diego, California) for 1 hour at room temperature. These plates were then washed 3 times with wash buffer (0.05% polysorbate 20 [Tween 20] in phosphate-buffered saline) and blotted on a paper towel. Next, the supernatants and eotaxin standards were added in triplicate and the plates were then incubated at room temperature for 2 hours. The contents of the wells were then discarded, after which the wells were washed 5 times with wash buffer. Next, avidin–horseradish peroxidase–conjugated anti-eotaxin antibodies were added to the wells and the plates were incubated for 1 hour at room temperature. The plates were then washed, after which tetramethylbenzidine substrate solution (Pharmingen) was added. The color was allowed to develop for 30 minutes in the dark before the reaction was quenched with 0.2M sulfuric acid stop solution (Sigma, St Louis, Missouri). The absorbance was then read at 450 nm using a microplate reader (Molecular Devices, Sunnyvale, California).

Eosinophil Chemotaxis Assays

A549 cells were stimulated by treatment with T4B for 24 hours, after which they were treated with *V rotundifolia* extract (0.1 or 1 μ g/mL) for 24 hours. The supernatants of the cultures were then used for a chemotaxis assay. Briefly, 56 μ L of EoL-1 cells (1×10^5 per milliliter) was added to the upper compartment of Transwell 12-well plates with 5- μ m polycarbonate membrane filters (Neuroprobe Inc, Gaithersburg, Maryland), whereas 28 μ L of supernatant was added to the bottom of the wells. The samples were then incubated for 90 minutes, after which the total numbers of migrated EoL-1 cells were counted.

RNA Preparation

A549 cells were initially cultured in a 100-mm dish (1×10^6 per milliliter) for 24 hours at 37°C, after which they were stimulated with T4B for 30 minutes. Next, 0 or 1 μ g/mL of *V rotundifolia* was added and the cells were then incubated at 37°C for 0.5, 1, 3, or 6 hours. The total RNA was then isolated from the A549 cells using a mini kit (Rneasy; Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions and subsequently quantified using a spectrophotometer (1000 NanoDrop; NanoDrop Technologies Inc, Wilmington, Delaware).

Oligonucleotide Chip Microarray Analysis

Microarray analysis was performed using single round RNA amplification protocols, following published specifications (Affymetrix GeneChip Expression Analysis Technical Manual). Briefly, 3 μ g of total RNA was used to synthesize first-strand complementary DNA (cDNA) using oligonucleotide probes with 24oligo-dT plus T7 promoter as primer (Proligo LLC, Boulder, Colorado) and the Superscript Choice System (Invitrogen Life Technologies). Double-stranded cDNA was then synthesized and purified by phenol-chloroform extraction, and biotinylated antisense complementary RNA (cRNA) was generated through in vitro transcription using a kit (BioArray RNA High-Yield Transcript Labeling kit; ENZO Life Sciences Inc, Farmingdale, New York). The biotinylated cRNA was then fragmented, and 10 μ g of total fragmented cRNA was hybridized to the Affymetrix Human Genome U133 Plus 2.0 GeneChip array (P/N900470; Affymetrix Inc, Santa Clara, California), washed, stained, and scanned according to the manufacturer's instructions. The fluorescence was detected using a scanner (Genechip System Confocal Scanner; Hewlett-Packard, Santa Clara), and analysis of each gene chip was conducted using GeneChip 3.1 software produced by Affymetrix Inc, using the standard default settings. To compare different chips, global scaling was used, with all probe sets being scaled to a user-defined target intensity of 150.

Statistical Analysis

The MAS5 algorithm was used to evaluate the expression signals generated by the gene chip. Global scaling normalization was then performed, and the normalized data were log transformed using base 2. Next, fold change was applied to

select the differentially expressed genes using a fold change threshold of 1.5-fold and a $P < .05$ to indicate statistical significance. Determined for probe sets with 16 to 20 probe pairs (defaults $\alpha_1 = .04$ and $\alpha_2 = .06$), any P value below α_1 is assigned a P (present call) and any P value above α_2 is assigned an A (absent call). M (marginal call) is given to probe sets that have a P value between α_1 and α_2 . Therefore, probe sets that resulted in A calls in the compared groups were removed to filter false-positive results. Gene ontology significance analysis was conducted to investigate the functional relationships among the 1.5-fold differentially expressed genes using high-throughput GoMiner (<http://discover.nci.nih.gov/gominer/>). The candidate gene list was selected by fold changes larger than 1.5-fold ($\log_2$).

RTRP Analysis

Microarray verification was performed by real-time reverse transcription–polymerase chain reaction (RTRP) analysis of selected genes using SYBR Green I Master Mix (Applied Biosystems, Foster City, California) and primers (Genotech Inc, Daejeon, Korea). cDNA was synthesized using 2 μ g of RNA in a reverse transcription reaction. RTRP quantitative messenger RNA (mRNA) analyses were performed with a real-time polymerase chain reaction system (model 7300; Applied Biosystems) using a fluorescence quantification system (SYBR Green; Applied Biosystems) to quantify the amplicons. The polymerase chain reaction conditions were 40 cycles of 95°C for 15 seconds, 60°C for 1 minute, and a standard denaturation curve. The sequences of the human primers were as follows: eotaxin (forward primer [FW]: 5'-cctccaacatgaaggtctcc-3'; reverse primer [RW]: 5'-gttaagcagcaggtgttg-3'), ICAM-1 (FW: 5'-tgaggagatc accatgagc-3'; RW: 5'-gccaggaacagaccacgt-3'), VCAM-1 (FW: 5'-gagaaccagatagacagccc-3'; RW: 5'-ttcacgaggccaccatcatc-3'), IL-8 (FW: 5'-atgactccaagctggcgtg-3'; RW: 5'-tgtgttggtggcagctgtgtgc-3'), nitric oxide synthase (NOS) 2A (FW: 5'-accaggaggaga tgctggag-3'; RW: 5'-aacatagaggtggcctggct-3'), IL-5RA (FW: 5'-cactggggagaaatcgca-3'; RW: 5'-agctgatcaaaggcctgat-3'), integrin $\alpha 7$ (ITGA7) (FW: 5'-ctgtgtggcagaaggagtg-3'; RW: 5'-atcccatctccacaggagc-3'), NOS1 (FW: 5'-cagccttttcattcttgcca-3'; RW: 5'-aggcttgtaggacatgtgg-3'), forkhead box P3 (FOXP3) (FW: 5'-gtgactgggatggcctcaa-3'; RW: 5'-caccaccaacaaccacatc-3'), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (FW: 5'-ttcaccaccatggagaaggc-3'; RW: 5'-ggcatggactgtgtcatga-3'). Polymerase chain reaction conditions for each target were optimized according to the primer concentration, the absence of primer dimer formation, and the efficiency of amplification of both the target genes and the housekeeping gene control. Polymerase chain reactions were performed in a total volume of 20 μ L in polymerase chain reaction master mix containing 10 μ L of 2X SYBR Green premix solution, 5 μ M each of sense and antisense primer, and 2 μ L of 1:2 diluted cDNA filled up to 20 μ L with diethyl pyrocarbonate–treated water. To normalize the cDNA content of the samples, we used the comparative threshold cycle method, which consists

of the normalization of the number of target gene copies vs the endogenous reference gene, *GAPDH*. The *comparative threshold* is defined as the fractional cycle number at which the fluorescence generated by cleavage of the probe passes a fixed threshold baseline when amplification of the polymerase chain reaction product is first detected.

Statistical analysis of the data was conducted using Prism 3.02 software (GraphicPad Software Inc, La Jolla, California). Data were analyzed by 1-way analysis of variance for multiple comparisons. Results with a $P < .05$ were considered statistically significant.

RESULTS

V rotundifolia Down-Regulates Eotaxin Secretion in A549 Cells

A549 cells were stimulated with T4B with *V rotundifolia* (0, 0.1, 1, or 10 μ g/mL). The minimum detectable eotaxin concentration was 6.3 pg/mL. Eotaxin secretion decreased significantly, by more than 80%, after treatment with 0.1 and 1 μ g/mL of *V rotundifolia* (Fig 1). Therefore, concentrations of 0.1 and 1 μ g/mL of *V rotundifolia* were used for the eosinophil chemotaxis assay.

V rotundifolia Inhibits Eosinophil Migration

A549 cells were stimulated by treatment with T4B with or without *V rotundifolia* (0.1 and 1 μ g/mL). The supernatants of the cultures were then used for a chemotaxis assay. Eosinophil migration was significantly inhibited by 1 μ g/mL of *V rotundifolia* (Fig 2); therefore, cells were treated with 1 μ g/mL of *V rotundifolia* before being analyzed by oligonucleotide microarray analysis.

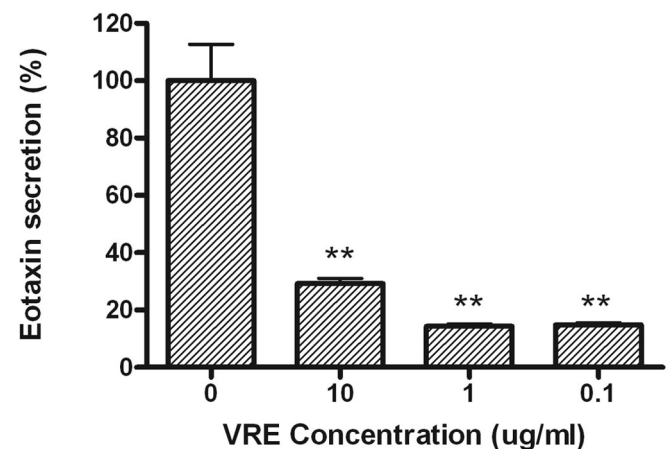


Figure 1. Inhibitory effects of *Vitex rotundifolia* (VRE) on eotaxin secretion from A549 cells stimulated with cytokines by enzyme-linked immunosorbent assay analysis. The A549 cells were stimulated with interleukin (IL) 4, IL-1 β , and tumor necrosis factor (TNF) α in the presence of the indicated amount of VRE. Eotaxin secretion is expressed as percentage of positive control (100% is the amount of eotaxin secreted from A549 cells treated with IL-4, IL-1 β , and TNF- α in the absence of VRE). Data are presented as mean (SEM) ($n = 3$). The minimum detectable concentration of eotaxin was 6.3 pg/mL. ** $P < .01$ compared with positive control.

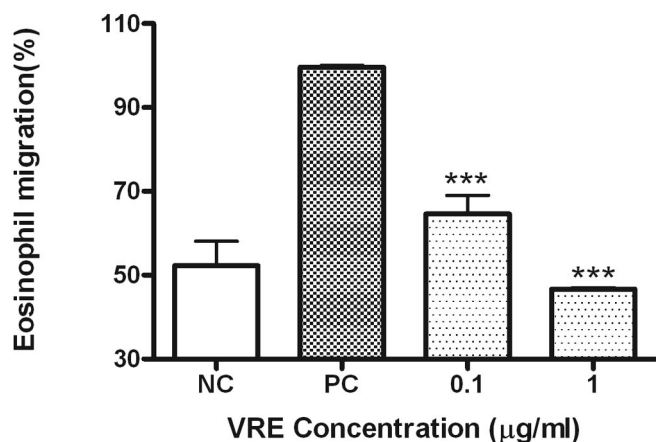


Figure 2. Effect of *Vitex rotundifolia* (VRE) on eosinophil migration. The EoL-1 eosinophil migration was assessed by the Transwell chemotaxis assay, as described in the “Methods” section. The EoL-1 cell migration is expressed as percentage of positive control (100% is the number of cells migrated toward the lower chamber containing supernatant of A549 cells treated with interleukin [IL] 4, IL-1 β , and tumor necrosis factor [TNF] α in the absence of VRE). Data are presented as mean (SEM) (n = 3). NC indicates negative control (media only); PC, positive control (IL-4, IL-1 β , and TNF- α -stimulated A549 cell). *** P < .001 compared with PC.

Gene Expression Profiles in A549 Cells

Gene expression profiles were significantly up- or down-regulated in the *V rotundifolia*-treated A549 cells when compared with control cells (stimulated with T4B without *V rotundifolia*) or negative control cells (not stimulated with T4B). For the *V rotundifolia*-treated cells, genes that had highly altered expression levels were aligned according to the magnitude of the altered expression. The up- and down-regulated genes are listed in Table 1. The cellular processes represented by these genes include adhesion molecules (*SELE*, *ITGB6*, *ITGB1*, *ITGA7*, *VCAM1*, and *ICAM1*), proinflammatory cytokines and receptors (*CSF2*, *IL1B*, *IL5RA*, and *IL8*), G-proteins (*GPR84*, *GPR39*, and *GRK4*), growth factors (*PDGFB*, *FGF5*, *FGF19*, *FGFR1*, *FGF18*, *FGF23*, and *EGFR*), and genes associated with the mitogen-activated protein kinase (MAPK) pathway (*MAP3K7IP2*, *MAP3K7IP1*, *MAPKAP1*, *MAP4K1*, and *MAP3K7IP1*).

Gene Expression Validation

The effects of *V rotundifolia* on A549 cells that were stimulated with T4B were evaluated using RTRP to measure changes in the mRNA levels of the eotaxin gene and of *ICAM*, *VCAM*, *IL8*, *NOS2A*, *IL5RA*, *ITGA7*, *NOS1*, and *FOXP3*. To normalize the expression-level changes, we used the expression level of the control gene, *GAPDH*, which is known to be invariant when treated with *V rotundifolia*. RTRP showed that the eotaxin gene and *ICAM*, *VCAM*, *IL8*, *NOS2A*, *IL5RA*, *ITGA7*, and *NOS1* mRNA increased significantly in stimulated A549 cells, whereas the mRNA levels of these genes were significantly lower in *V rotundifolia*-treated A549 cells. In addition, RTRP revealed significant increases

in *FOXP3* gene expression in *V rotundifolia*-treated A549 cells. The mRNA levels of *ICAM* and *IL-8* were particularly sensitive to *V rotundifolia* treatment: their levels were 40% lower in *V rotundifolia*-treated cells than in control cells (Fig 3B and D).

DISCUSSION

Natural products have long been used in traditional medicine to treat asthma and other allergic diseases; recently, the raw materials used to produce such products have been used to develop new drugs.^{8,11} The purpose of this study was to determine how treatment with *V rotundifolia* affected the gene expression profiles of human epithelial lung cells (A549). To accomplish this, A549 cells were stimulated with T4B with or without *V rotundifolia*. Proinflammatory cytokines, such as TNF- α and IL-1 β , are key regulators of immune and inflammatory genes,¹² and IL-4 is a pivotal cytokine that is produced by T_H2 cells and associated with allergic airway diseases that support T_H2 development.¹³

Eotaxin is of particular interest in asthma because it can be detectable in airway epithelial cells and its presence in such cells is increased in asthma.¹² In this study, we found that treatment with *V rotundifolia* (0.1, 1, and 10 μ g/mL) reduced eotaxin secretion and gene expression by A549 cells that had been stimulated by treatment with cytokines (Fig 1 and Fig 3A). Eotaxin is also associated with the degree of subepithelial fibrosis and eosinophilic airway inflammation.¹⁴ In addition, eosinophil recruitment into the lung is associated with allergic airway diseases.^{15,16} In general, increases in the number of eosinophils in the tissues, blood, and bone marrow correlate with the severity of the asthma.¹⁷ In addition, eosinophil migration into specific tissues is a hallmark of allergic diseases. Consequently, we performed an eosinophil chemotaxis assay to determine if *V rotundifolia* inhibited eosinophil migration. The results of the assay revealed that eosinophil migration was significantly inhibited by treatment with 1 μ g/mL of *V rotundifolia* (Fig 2). Therefore, 1 μ g/mL of *V rotundifolia* was used for subsequent studies to evaluate the mechanisms by which it exerted its effects on T4B-induced epithelial lung cell activation. We also demonstrated that *V rotundifolia* did not affect the viability of A549 cells (data not shown). However, the early signaling events involved in the T4B-induced activation of epithelial lung cells are not completely understood; therefore, we evaluated the gene expression profiles of A549 cells that were treated with *V rotundifolia* for different lengths of time (0.5, 1, 3, and 6 hours).

In many inflammatory situations, the expression of adhesion molecules that are known to play important roles in the induction of airway inflammation is induced by cytokines. For example, the interaction of the adhesion molecules on vascular endothelial cells (eg, *ICAM1*, *VCAM1*, and E selectin) with their counterreceptors on circulating leukocytes (eg, β 1 and β 2 integrins) results in the capture, rolling, and firm adhesion of the leukocytes to the vascular endothelium. The arrested leukocytes then transigrate the vascular wall

Table 1. Up- and Down-Regulation of Genes Based on Comparison of Gene Expression Between *Vitex rotundifolia*-Treated A549 Cells and Positive or Negative Control^a

Gene		Regulation profile and ratio ^b							
Description	Abbreviation	Negative vs positive control				Positive control vs <i>V rotundifolia</i>			
		0.5 h	1 h	3 h	6 h	0.5 h	1 h	3 h	6 h
Inflammatory and Immune Response–Related Genes									
Colony-stimulating factor 2 (granulocyte-macrophage)	<i>CSF2</i>	6.6	7.5	9.9	7.3	0.5	−0.1	−0.1	−0.2
Chemokine (C-X-C motif) ligand 2	<i>CXCL2</i>	6.0	6.1	5.9	6.0	0.7	−0.4	0.6	−0.5
Chemokine (C-C motif) ligand 20	<i>CCL20</i>	5.6	5.6	6.2	5.5	−0.0	0.03	−0.3	−0.0
Proplatelet basic protein (chemokine [C-X-C motif] ligand 7)	<i>PPBP</i>	4.4	4.0	4.0	4.4	−1.6	−1.1	−0.3	−0.5
Chemokine (C-C motif) ligand 14	<i>CCL14</i>	4.3	1.0	5.2	4.3	−0.4	1.24	−1.2	−1.2
Chemokine (C-X-C motif) ligand 3	<i>CXCL3</i>	3.8	4.1	5.1	4.7	0.1	−0.1	−0.2	−0.1
Chemokine (C-C motif) ligand 8	<i>CCL8</i>	3.6	2.2	5.3	5.4	−0.5	−2.1	0.4	0.3
Interleukin 8	<i>IL8</i>	3.5	3.6	4.1	3.6	−0.0	−0.1	−0.1	−0.2
Interleukin 1 α	<i>IL1A</i>	3.4	4.0	5.9	3.5	0.0	−0.2	0.0	0.0
Interleukin 1 receptor antagonist	<i>IL1RN</i>	3.4	3.2	2.8	4.3	−0.8	−1.7	0.1	−0.7
Interleukin 1 receptor antagonist	<i>IL1RN</i>	3.3	0.7	1.9	0.4	−3.2	−1.1	−2.4	−0.9
Forkhead box P3	<i>FOXP3</i>	−3.0	−2.0	−2.0	−3.0	0.8	1.3	−0.7	3.1
Interleukin 26	<i>IL26</i>	0.4	2.6	5.0	5.1	1.8	−1.1	−2.9	−2.8
Fc fragment of IgE, high-affinity I, receptor for γ polypeptide	<i>FCER1G</i>	0.4	0.7	0.7	1.5	−2.8	−2.4	−1.2	−3.1
Chemokine (C-X-C motif) ligand 14	<i>CXCL14</i>	0.5	0.4	2.2	2.0	2.3	−0.8	−1.4	−0.8
Chemokine (C-C motif) ligand 7	<i>CCL7</i>	0.7	1.3	2.4	2.5	−0.6	−4.6	−0.9	−0.8
Chemokine (C-C motif) ligand 5	<i>CCL5</i>	0.8	2.4	7.0	7.5	0.2	−0.9	−0.2	0.2
Interleukin 17C	<i>IL17C</i>	0.8	1.6	1.7	−0.4	−1.3	−2.3	−1.1	−0.3
Tumor necrosis factor receptor superfamily, member 9	<i>TNFRSF9</i>	0.8	2.0	3.8	3.9	0.6	−0.3	−0.1	−0.2
Chemokine (C-X-C motif) receptor 4	<i>CXCR4</i>	1.1	1.2	1.9	2.2	−0.7	−0.2	0.1	−0.2
Interleukin 1 β	<i>IL1B</i>	1.7	2.1	6.1	5.2	0.2	−0.3	0.1	−0.2
Interleukin 1 receptorlike 1	<i>IL1RL1</i>	2.4	2.6	2.0	2.2	0.1	−0.5	−0.4	0.5
Chemokine (C-X-C motif) ligand 1	<i>CXCL1</i>	2.8	3.3	4.7	4.7	0.1	−0.0	0.0	−0.1
Interleukin 6 (interferon β 2)	<i>IL6</i>	2.7	3.2	3.9	4.3	0.3	−0.1	0.0	−0.3
Chemokine (C-X-C motif) ligand 2	<i>CXCL2</i>	2.6	2.5	2.7	2.3	−0.0	0.1	−0.1	−0.0
Chemokine (C-C motif) ligand 23	<i>CCL23</i>	2.5	3.1	1.6	2.9	−2.1	−2.4	0.2	0.0
Chemokine (C-C motif) receptor 4	<i>CCR4</i>	0.4	2.3	2.3	3.1	2.5	−1.2	−2.7	−0.8
Interleukin 5 receptor α	<i>IL5RA</i>	4.2	0.8	1.5	3.2	−3.7	−0.1	−0.6	−1.7
Signal Transduction–Related Genes									
Mitogen-activated protein kinase kinase kinase 7 interacting protein 2	<i>MAP3K7IP2</i>	3.9	3.7	4.4	3.8	0.5	−0.2	−0.4	−0.0
Tumor necrosis factor α –induced protein 3	<i>TNFAIP3</i>	3.9	4.4	4.5	4.0	−0.0	−0.1	0.2	0.1
Protein kinase C, β 1	<i>PRKCB1</i>	3.8	5.2	3.1	5.5	0.5	−1.0	−1.9	−3.7
G protein–coupled receptor 84	<i>GPR84</i>	3.4	3.0	5.1	4.6	−0.3	−2.9	−0.9	−2.5
Vasoactive intestinal peptide receptor 2	<i>VIPR2</i>	0.7	1.1	0.7	2.1	−1.9	−1.2	−1.6	−0.7
Mitogen-activated protein kinase–associated protein 1	<i>MAPKAP1</i>	2.5	2.9	1.5	3.1	−2.0	−3.4	−0.6	−0.6
Mitogen-activated protein kinase kinase kinase 1	<i>MAP4K1</i>	2.5	1.6	1.1	−0.1	−3.3	−2.0	−1.2	−0.9
Glutamate receptor, metabotropic 7	<i>GRM7</i>	2.3	2.3	2.1	2.2	−1.7	−2.0	−0.8	−3.0
Mitogen-activated protein kinase kinase kinase 7 interacting protein 1	<i>MAP3K7IP1</i>	2.2	1.1	0.9	0.8	−2.5	−1.2	−1.0	−1.6
G protein–coupled receptor 39	<i>GPR39</i>	2.1	2.6	1.6	2.7	0.2	−0.8	0.5	−2.5
G protein–coupled receptor kinase 4	<i>GRK4</i>	1.9	4.4	5.1	3.9	1.8	−3.5	−4.4	−0.0

^a The A549 cells and the positive control were stimulated with tumor necrosis factor α , interleukin (IL) 4, and IL-1 β ; the positive control was not treated with *V rotundifolia*.

^b Ratio: log 2 (fold change).

Table 1. Continued

Gene		Regulation profile and ratio ^b							
Description	Abbreviation	Negative vs positive control				Positive control vs <i>V rotundifolia</i>			
		0.5 h	1 h	3 h	6 h	0.5 h	1 h	3 h	6 h
Growth Factor–Related Genes									
Fibroblast growth factor 5	<i>FGF5</i>	3.2	3.3	3.2	0.1	−0.5	−1.1	−0.5	0.3
Fibroblast growth factor 19	<i>FGF19</i>	−0.1	−0.1	−0.3	2.1	−1.4	−1.9	−1.5	−2.3
Fibroblast growth factor receptor 1	<i>FGFR1</i>	0.7	1.6	0.8	1.1	0.5	−0.6	−0.4	0.1
Fibroblast growth factor 18	<i>FGF18</i>	1.6	1.7	4.8	3.3	−0.5	−2.9	−0.0	−0.4
Fibroblast growth factor 23	<i>FGF23</i>	1.6	1.2	2.1	2.2	0.2	−0.3	−1.7	−1.6
Platelet-derived growth factor β polypeptide	<i>PDGFB</i>	1.8	1.2	1.0	1.1	−3.7	−3.2	−3.2	−1.0
Epidermal growth factor receptor	<i>EGFR</i>	2.2	2.9	0.4	2.5	0.1	−2.2	−1.3	−0.3
Proteolysis-Related Genes									
ADAM metallopeptidase domain 10	<i>ADAM10</i>	3.7	3.7	2.4	3.7	−0.8	−0.8	−1.3	0.3
Matrix metallopeptidase 26	<i>MMP26</i>	3.5	3.2	4.0	3.5	−0.4	0.7	−0.7	−0.2
ADAM metallopeptidase domain 12 (meltrin α)	<i>ADAM12</i>	0.7	2.3	2.0	2.5	−1.1	−0.6	−1.3	−1.0
Matrix metallopeptidase 15 (membrane inserted)	<i>MMP15</i>	1.5	1.8	2.9	2.2	1.2	−0.6	−4.0	−3.1
Matrix metallopeptidase 3 (stromelysin 1, progelatinase)	<i>MMP3</i>	2.0	2.8	5.4	5.5	−0.0	−0.3	0.2	0.2
ADAM metallopeptidase with thrombospondin type 1 motif, 8	<i>ADAMTS8</i>	2.1	2.2	2.1	0.4	−2.0	−0.9	−1.9	−0.7
Cell Adhesion–Related Genes									
Selectin E (endothelial adhesion molecule 1)	<i>SELE</i>	4.6	5.5	7.5	5.7	−0.2	−0.2	0.1	0.3
Integrin, $\beta 6$	<i>ITGB6</i>	−0.2	0.2	0.8	0.5	0.3	−0.8	−0.6	−1.0
Immunoglobulin superfamily, member 4	<i>IGSF4</i>	0.8	1.6	2.6	3.3	2.9	−1.0	−1.1	−1.0
Integrin, $\beta 1$	<i>ITGB1</i>	0.9	1.4	1.5	1.8	−0.1	−0.8	−0.4	−0.8
Vascular cell adhesion molecule 1	<i>VCAM1</i>	1.0	1.8	3.5	3.9	−0.5	−1.8	−0.1	−0.7
Intercellular adhesion molecule 1	<i>ICAM1</i>	2.0	3.1	6.6	5.3	0.5	0.0	−0.1	0.0
Integrin α FG-GAP repeat containing 2	<i>ITFG2</i>	2.0	2.4	2.7	3.0	−3.0	−1.2	−1.9	−3.1
Tumor necrosis factor receptor superfamily, member 9	<i>TNFRSF9</i>	2.3	2.3	4.1	5.1	−0.9	0.2	0.2	−1.4
Integrin $\alpha 7$	<i>ITGA7</i>	2.4	1.4	1.2	1.1	−0.5	−1.5	−2.6	−2.8
Transport-Related Gene									
Nitric oxide synthase 1 (neuronal)	<i>NOS1</i>	0.4	0.7	0.6	0.2	−0.1	−2.3	−1.4	−2.1
Nitric oxide synthase 3 (endothelial cell)	<i>NOS3</i>	0.7	2.3	2.3	2.7	1.8	−0.4	−0.6	−0.0
Cytochrome P450, family 2, subfamily E, polypeptide 1	<i>CYP2E1</i>	1.3	1.1	0.8	2.1	−0.4	−2.0	−1.0	−1.3
Nitric oxide synthase 2A (inducible, hepatocytes)	<i>NOS2A</i>	2.6	2.9	3.0	3.5	−0.9	−2.8	0.8	−0.9
Cytochrome P450, family 4, subfamily V, polypeptide 2	<i>CYP4V2</i>	2.9	1.9	1.3	3.4	−1.5	−1.9	0.2	−4.1

and move toward the lesion along the chemotactic gradient.^{18–21} In this study, adhesion molecules were down-regulated in *V rotundifolia*-treated A549 cells (Table 1 and Fig 3B, C, and G).

MAPKs are involved in signaling that leads to the synthesis of proinflammatory cytokines and chemokines.²² However, the exact role of proinflammatory cytokines and receptors, such as *CSF2*, *IL1B*, *IL5RA*, and *IL8*, which regulate cell behavior, including growth, secretion, and migration in physiologic and pathologic conditions, is not known. Increased levels of these cytokines and receptors have been observed in the asthmatic airway epithelium^{15,23,24}; in this study, we detected the down-regulation of proinflammatory cytokines and receptors in *V rotundifolia*-treated A549 cells (Table 1 and Fig 3D and F). MAPKs are serine/threonine kinases that

include the extracellular signal-related kinases, p38 kinases, and c-Jun N-terminal kinases. Activation of the MAPK pathway often occurs in response to growth factor stimulation of receptor tyrosine kinases, which are coupled to the activation of Ras G-proteins through Src homology 2 domain-containing proteins, such as Shc and Grb2, and guanine nucleotide exchange factors, such as SOS.^{25,26} In this study, we detected the down-regulation of the MAPK pathway, G proteins, and growth factor in *V rotundifolia*-treated A549 cells (Table 1). Each MAPK has also been implicated in inflammatory events, including mediation of many of the physiologic responses to nitric oxide (NO). For example, NO regulation of matrix metalloproteinases proteins, including MMP1, during inflammatory and angiogenic responses may require MAP kinase proteins. In this study, *MMP3*, *MMP15*, and *MMP26*

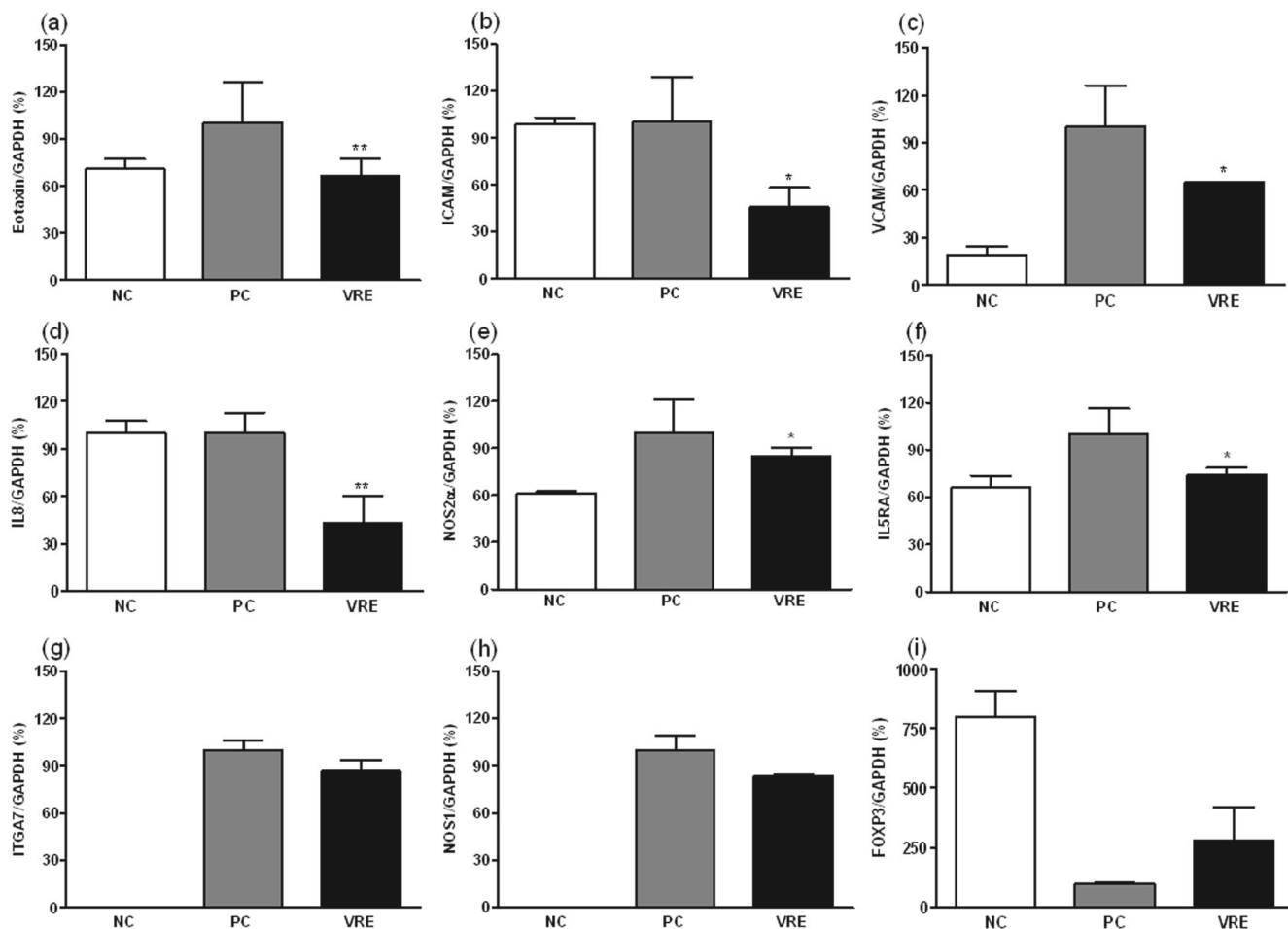


Figure 3. Effects of *Vitex rotundifolia* (VRE) on messenger RNA levels in activated A549 cells seen by real-time reverse transcription–polymerase chain reaction: eotaxin (A), intercellular adhesion molecule (ICAM) (B), vascular cell adhesion molecule (VCAM) (C), interleukin (IL) 8 (D), nitric oxide synthase (NOS) 2A (E), IL-5RA (F), integrin $\alpha 7$ (ITGA7) (G), NOS1 (H), and forkhead box P3 (FOXP3) (I). The positive control (PC) was IL-4, IL-1 β , and TNF- α -stimulated A549 cell. Data are presented as mean (SEM) (n = 3). * $P < .05$ and ** $P < .01$ compared with PC. GAPDH indicates glyceraldehyde-3-phosphate dehydrogenase; NC, negative control.

were down-regulated in *V rotundifolia*-treated A549 cells (Table 1). NO is a reactive free radical that generally increases during inflammatory airway diseases such as asthma.^{25,27,28} In this study, we found that the mRNA levels of NOS1, NOS2A, and NOS3 were down-regulated in *V rotundifolia*-treated A549 cells (Table 1 and Fig 3E and H).

The results of this study demonstrated that *FoxP3* expression was inhibited on T4B treatment, but this suppression was alleviated by *V rotundifolia* treatment (Table 1 and Fig 3I). *FoxP3* may be a master regulatory gene for the function of regulatory T cells (Treg) in humans and rodents. In addition, *FoxP3*, which encodes a forkhead/winged-helix transcription repressor, has been shown to be specifically expressed in Treg cells and associated with their development and function. Mutations of the *FoxP3* gene lead to a loss of Treg cells and underlie a homologous autoimmune lymphoproliferative

disorder that includes allergic dysregulation syndrome. Furthermore, *FoxP3* transfection into Jurkat T cells resulted in impaired proliferation and production of cytokines, including IL-2 and IFN- γ , on stimulation, while suppressing the in vitro proliferation of other T cells in a cell-cell contact-dependent manner.^{29–31} However, the role of *FoxP3* in cells other than Treg is mostly unknown. Indeed, it has only recently been reported that *FoxP3* is expressed outside lymphoid tissue, and its function in these cells is completely unknown.³² To the best of our knowledge, this study provides the first confirmative evidence that *FoxP3* expression may be regulated in *V rotundifolia*-treated lung epithelial cells.

In summary, the results of this study revealed that T4B induced the expression and secretion of inflammatory cytokines in A549 cells and that these cytokines were significantly inhibited by treatment with *V rotundifolia*. In addition,

V rotundifolia down-regulates the genes related with the MAPK pathway in activated A549 cells. Taken together, these results indicate that *V rotundifolia* may have potential efficacy for the treatment of inflammatory disease through anti-inflammatory activity that occurs via inhibition of the inflammatory cytokines and the MAPK pathway.

REFERENCES

- Newton R, Holden NS, Catley MC, et al. Repression of inflammatory gene expression in human pulmonary epithelial cells by small-molecule I κ B kinase inhibitors. *J Pharmacol Exp Ther*. 2007;321:734–742.
- Hughes JM, Arthur CA, Baracho S, et al. Human eosinophil-airway smooth muscle cell interactions. *Mediators Inflamm*. 2000;9:93–99.
- Mochizuki M, Bartels J, Mallet AI, Christophers E, Schroder JM. IL-4 induces eotaxin: a possible mechanism of selective eosinophil recruitment in helminth infection and atopy. *J Immunol*. 1998;160:60–68.
- Bochner BS, Klunk DA, Sterbinsky SA, Coffman RL, Schleimer RP. IL-13 selectively induces vascular cell adhesion molecule-1 expression in human endothelial cells. *J Immunol*. 1995;154:799–803.
- Sekiya T, Miyamasu M, Imanishi M, et al. Inducible expression of a Th2-type CC chemokine thymus- and activation-regulated chemokine by human bronchial epithelial cells. *J Immunol*. 2000;165:2205–2213.
- Moore PE, Church TL, Chism DD, Panettieri RA Jr, Shore SA. IL-13 and IL-4 cause eotaxin release in human airway smooth muscle cells: a role for ERK. *Am J Physiol Lung Cell Mol Physiol*. 2002;282:L847–L853.
- Bentley AM, Durham SR, Robinson DS, et al. Expression of endothelial and leukocyte adhesion molecules intercellular adhesion molecule-1, E-selectin, and vascular cell adhesion molecule-1 in the bronchial mucosa in steady-state and allergen-induced asthma. *J Allergy Clin Immunol*. 1993;92:857–868.
- Hu Y, Hou TT, Xin HL, et al. Estrogen-like activity of volatile components from *Vitex rotundifolia* L. *Indian J Med Res*. 2007;126:68–72.
- Okuyama E, Fujimori S, Yamazaki M, Deyama T. Pharmacologically active components of viticis fructus (*Vitex rotundifolia*), II: the components having analgesic effects. *Chem Pharm Bull (Tokyo)*. 1998;46:655–662.
- Shin TY, Kim SH, Lim JP, et al. Effect of *Vitex rotundifolia* on immediate-type allergic reaction. *J Ethnopharmacol*. 2000;72:443–450.
- Kim J, Lee H, Lee Y, et al. Inhibition effects of Moutan Cortex Radicis on secretion of eotaxin in A549 human epithelial cells and eosinophil migration. *J Ethnopharmacol*. 2007;114:186–193.
- Lilly CM, Nakamura H, Kesselman H, et al. Expression of eotaxin by human lung epithelial cells: induction by cytokines and inhibition by glucocorticoids. *J Clin Invest*. 1997;99:1767–1773.
- Matsukura S, Kokubu F, Kurokawa M, et al. Molecular mechanisms of repression of eotaxin expression with fluticasone propionate in airway epithelial cells. *Int Arch Allergy Immunol*. 2004;134(suppl 1):12–20.
- Rankin SM, Conroy DM, Williams TJ. Eotaxin and eosinophil recruitment: implications for human disease. *Mol Med Today*. 2000;6:20–27.
- Cheng G, Ueda T, Eda F, Arima M, Yoshida N, Fukuda T. A549 cells can express interleukin-16 and stimulate eosinophil chemotaxis. *Am J Respir Cell Mol Biol*. 2001;25:212–218.
- Liu LY, Mathur SK, Sedgwick JB, Jarjour NN, Busse WW, Kelly EA. Human airway and peripheral blood eosinophils enhance Th1 and Th2 cytokine secretion. *Allergy*. 2006;61:589–597.
- Kay AB. The role of eosinophils in the pathogenesis of asthma. *Trends Mol Med*. 2005;11:148–152.
- Lee KS, Jin SM, Kim HJ, Lee YC. Matrix metalloproteinase inhibitor regulates inflammatory cell migration by reducing ICAM-1 and VCAM-1 expression in a murine model of toluene diisocyanate-induced asthma. *J Allergy Clin Immunol*. 2003;111:1278–1284.
- Sabatini F, Silvestri M, Sale R, et al. Fibroblast-eosinophil interaction: modulation of adhesion molecules expression and chemokine release by human fetal lung fibroblasts in response to IL-4 and TNF- α . *Immunol Lett*. 2002;84:173–178.
- Zhu GD, Arendsen DL, Gunawardana IW, et al. Selective inhibition of ICAM-1 and E-selectin expression in human endothelial cells, 2: aryl modifications of 4-(aryloxy)thieno[2,3-c]pyridines with fine-tuning at C-2 carbamides. *J Med Chem*. 2001;44:3469–3487.
- Zerfaoui M, Suzuki Y, Naura AS, Hans CP, Nichols C, Boulares AH. Nuclear translocation of p65 NF- κ B is sufficient for VCAM-1, but not ICAM-1, expression in TNF-stimulated smooth muscle cells: differential requirement for PARP-1 expression and interaction. *Cell Signal*. 2008;20:186–194.
- Bhattacharyya A, Pathak S, Datta S, Chattopadhyay S, Basu J, Kundu M. Mitogen-activated protein kinases and nuclear factor- κ B regulate *Helicobacter pylori*-mediated interleukin-8 release from macrophages. *Biochem J*. 2002;368:121–129.
- Gilmour J, Cousins DJ, Richards DF, Sattar Z, Lee TH, Lavender P. Regulation of GM-CSF expression by the transcription factor c-Maf. *J Allergy Clin Immunol*. 2007;120:56–63.
- Koch A, Knobloch J, Dammhayn C, et al. Effect of bacterial endotoxin LPS on expression of INF- γ and IL-5 in T-lymphocytes from asthmatics. *Clin Immunol*. 2007;125:194–204.
- Raines KW, Cao GL, Porsuphatana S, Tsai P, Rosen GM, Shapiro P. Nitric oxide inhibition of ERK1/2 activity in cells expressing neuronal nitric-oxide synthase. *J Biol Chem*. 2004;279:3933–3940.
- Sim S, Yong TS, Park SJ, et al. NADPH oxidase-derived reactive oxygen species-mediated activation of ERK1/2 is required for apoptosis of human neutrophils induced by *Entamoeba histolytica*. *J Immunol*. 2005;174:4279–4288.
- Akundi RS, Candelario-Jalil E, Hess S, et al. Signal transduction pathways regulating cyclooxygenase-2 in lipopolysaccharide-activated primary rat microglia. *Glia*. 2005;51:199–208.
- Zaragoza C, Soria E, Lopez E, et al. Activation of the mitogen activated protein kinase extracellular signal-regulated kinase 1 and 2 by the nitric oxide-cGMP-cGMP-dependent protein kinase axis regulates the expression of matrix metalloproteinase 13 in vascular endothelial cells. *Mol Pharmacol*. 2002;62:927–935.
- Chinen J, Shearer WT. Advances in asthma, allergy and immunology series 2004: basic and clinical immunology. *J Allergy Clin Immunol*. 2004;114:398–405.
- Choi BM, Pae HO, Jeong YR, Kim YM, Chung HT. Critical role of heme oxygenase-1 in Foxp3-mediated immune suppression. *Biochem Biophys Res Commun*. 2005;327:1066–1071.
- Wu K, Bi Y, Sun K, Xia J, Wang Y, Wang C. Suppression of allergic inflammation by allergen-DNA-modified dendritic cells depends on the induction of Foxp3+ regulatory T cells. *Scand J Immunol*. 2008;67:140–151.
- Chen GY, Chen C, Wang L, Chang X, Zheng P, Liu Y. Cutting Edge: broad expression of the FoxP3 locus in epithelial cells: a caution against early interpretation of fatal inflammatory diseases following in vivo depletion of FoxP3-expressing cells. *J Immunol*. 2008;180:5163–5166.

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