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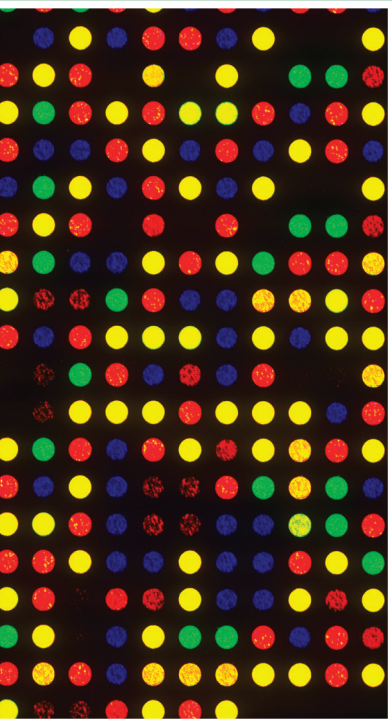
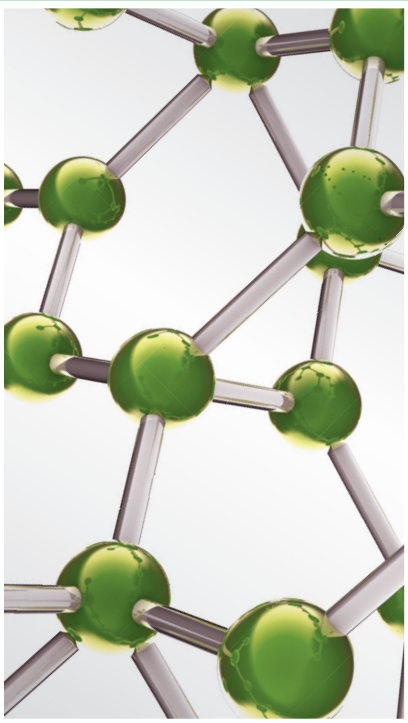
Guest Editors: Alexandre de Paula Rogerio, Troy Carlo, and Sergio R. Ambrosio



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Editorial Board

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Editorial

Bioactive Natural Molecules and Traditional Herbal Medicine in the Treatment of Airways Diseases

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The use of herbal medicines has been documented throughout recorded history including Assyrian clay tablets (2000 BC) and the Egyptian Ebers Papyrus (1550 BC) and in Ayurveda works from 900 BC. In addition, Hippocrates, Dioscorides, Galen, Paracelsus, and Arab scholars in Europe kept detailed notes regarding the use of medicinal plants. In the modern era, the research of natural bioactive molecules began with the isolation of morphine from opium latex in 1805 by Sertürner. A famous bioactive compound isolated from plants, salicylic acid, was isolated from willow bark in the 19th century. In 1900, Bayer patented a derivative of the compound to treat pain and inflammation and sold it under the brand name aspirin. Numerous other natural compounds have been discovered and used for the treatment of diseases.

Considerable progress has been made in the understanding of genetic and immunological factors that contribute to the development of airway disorders such as asthma, chronic obstructive pulmonary disease, and acute lung injury. Unfortunately, current therapies and treatments for airway disorders have not advanced dramatically despite our increased knowledge base. Traditional herbal medicine and bioactive natural products are being used as therapeutic substitutes or as complementary treatments to augment existing therapies. The natural compounds found in these may have the potential to form the basis of new drugs for the treatment of diseases. In this special issue, we took a particular interest in reviews and original papers that investigate the effects of bioactive natural molecules and traditional herbal medicines in clinical and preclinical (cellular or animal models) studies in prevention and treatment of airways diseases.

Allergic asthma is an airway inflammatory disorder coordinated by $CD4^+$ T cells with Th2 phenotype. The main characteristics of asthma are airway hyperresponsiveness, eosinophilic inflammation, and hypersecretion of mucus. Typical asthma treatments include β_2-agonists and inhaled or systemic corticosteroids. In addition, inhibitors of LT synthesis (such as zileuton, which directly inhibits 5-LO) or CysLT1 antagonists (such as montelukast, zafirlukast, and pranlukast) may also be used as complementary therapies to treat asthma, reducing the requirement for corticosteroids. Although the drugs described above have potent effects when used individually or in combination, they also have adverse side effects that limit their long-term use. Thus, it is necessary to develop new compounds with similar therapeutic potential and less adverse effects for the continuous treatment of airway diseases. In an ongoing search for bioactive plant-derived natural products, several groups, including some of this special issue, have successfully employed experimental methods to screen plant extracts and isolate compounds for pharmacological activity.

Ferulic acid (4-hydroxy-3-methoxycinnamic acid) is found in a wide array of plants, including fruits and vegetables, and in beverages such as coffee and beer. Ferulic acid demonstrates antioxidant and anti-inflammatory activities suggesting a therapeutic potential to treat several diseases such as arthritis and Alzheimer's disease.

Using a classical allergic airway inflammation model in mice (induced by ovalbumin) C.-C. Lee et al. demonstrated ferulic acid reduced most phenotypes associated with asthma including IgE and IgG1 production, airway hyperactivity, airway inflammation, chemokine (eotaxin), and cytokines (IL-4, IL-5, IL-13, and TNF-α) amounts in bronchoalveolar lavages. In addition, ferulic acid increased IFN-γ (an inhibitor of Th2 immune response) and IL-12 (inductor of differentiation of naive T cells toward the Th1 phenotype) production as well as reduced proinflammatory cytokines (TNF-α, IL-1β, and IL-6) production by lipopolysaccharide-stimulated bone-marrow-derived dendritic cells. This effect was associated with the ability of ferulic acid polarize Th1 cells (increasing IFN-γ) increasing expression level of Notch ligand Delta-like 4 (Dll4) and reducing the Jagged1 mRNA synthesis by LPS-stimulated dendritic cells. In addition, expression of MHC class II and CD40 molecules during dendritic cells maturation was increased. In a mixed lymphocyte reaction assay, ferulic acid induces proliferation of T cells with an associated increase of IFN-γ and reduction of IL-5 suggesting that ferulic acid possesses an antiallergic effect by restoring Th1/Th2 imbalance by modulating dendritic cells function.

Elaeagnus pungens leaves, a flowering plant from the Elaeagnaceae family, are used in traditional Chinese medicine to treat severe asthma, cough, bronchitis, or other respiratory disorders. Y. Ge et al. evaluated the role of aqueous extracts of *E. lanceolata*, *E. henryi*, and *E. pungens* to alleviate symptoms in an asthma model (induced by mixture spray of histamine and acetylcholine chloride) in guinea pigs, a tussive model (induced by exposure to aqueous ammonia) in mice, and an expectorant assay (measurement of the concentration of phenol red photometrically in whole trachea dissected from animals) in mice. All extracts demonstrated antiasthmatic, antitussive, and expectorant activities. These effects were associated with presence of flavonoids compounds in the leaves.

People with antigen-specific IgE antibodies and mast cells dependent allergic rhinitis can experience early phase symptoms (such as sneezing and rhinorrhea) within minutes of exposure to the associated allergens. However, such individuals also typically develop the nasal obstruction in late phase symptoms.

Alternative therapies such as aromatherapy have become more commonly used in a wide range of health problems including allergic rhinitis patients. Aromatherapy uses purified oils from fragrant plants to help relieve health problems and improve quality of life in general.

S. Y. Choi and K. Park evaluated the ability of aromatherapy using essential oils obtained from sandalwood, *Geranium*, and *Ravensara* to alleviate the symptoms perennial allergic rhinitis in adults. They demonstrated the aromatherapy improved total nasal symptom score, especially in nasal obstruction improving quality of life and fatigue in patients with perennial allergic rhinitis.

Smooth muscle cells play a significant role in the pathogenesis of asthma and are associated with bronchoconstriction, bronchial hyperresponsiveness, inflammation, and airway remodeling. *Cortex phellodendri*, an herb referred to as Huang Bai, is used in traditional Chinese medicines to treat dysentery, jaundice, urinary infection, and rheumatoid arthritis. Berberine, an alkaloid, is the major active constituent of *C. phellodendri*. Berberine was also isolated from *Argemone ochroleuca* (Papaveraceae) and demonstrated relaxant effect on guinea-pig tracheal smooth muscle.

Using an allergic inflammation model in mice (induced by ovalbumin) Q.-J. Jiang et al. were able to show that the *n*-butyl alcohol extract isolated from *C. phellodendri* (which does not contain berberine) inhibited high K^+- and acetylcholine-induced precontraction of airway smooth muscle in tracheal rings from control and asthmatic mice. In addition, similar results were obtained from lung slices taken from these mice. In follow-up experiments using nifedipine (L-voltage-sensitive Ca_2^+ channel blocker) and Pyr 3 (an TRPC3 and Orai1 channels blocker) the authors were able to demonstrate that tracheal rings relaxation responses were mediated by L-type Ca_2^+, TRPC3, and/or Orai1 channels. The authors suggest that *n*-butyl alcohol extract of *C. phellodendri* could be used in developing new drug for relieving bronchospasm.

Respiratory syncytial virus (RSV) is the most common cause of hospitalization in infants and young children in the world. Currently, there are several potential treatments for RSV infection in development (including vaccines and therapeutic agents). To date, there is no effective treatment for RSV lower respiratory tract infection. L. L. Lin et al. make a review of traditional Chinese medicine as well as bioactive compounds present in aqueous extracts and formulas derived from traditional Chinese medical herbs with potential to be used in the prevention and treatment of RSV.

Acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) are life-threatening syndromes that cause high morbidity and mortality. They can be caused directly by lung diseases such as such as pneumonia or aspiration of gastric contents or indirectly by systemic diseases with sepsis, burns, and pancreatitis serving as examples. There is a great therapeutic need for agents that have a clear benefit in ALI/ARDS treatment. ALI is characterized by alveolar edema and uncontrolled neutrophil migration to the lung. Unwanted neutrophil activation can lead to tissue damage through the release of proteases, oxidants, and cationic peptides.

Tanreqing injection (TRQ), a water-soluble natural extract from five traditional Chinese medicines, *Scutellariae baicalensis*, *Fel selenarcti*, *Cornu naemorhedi*, *Flos lonicerae*, and *Forsythiae fructus*, has demonstrated antibacterial, antiviral, and anti-inflammatory effects. W. Liu et al. using an acute lung injury model in rats induced by LPS demonstrated that TRQ, administrated by intraperitoneal injection, decreased airways inflammation, mucus production, and proinflammatory cytokines (TNF-α, IL-1β, IL-6, and IL-8) via regulation of NF-κB, ERK1/2, JNK, and p38 MAPK pathways.

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We encourage and appreciate your further support for this annual/special issue series.

Alexandre de Paula Rogerio
Troy Carlo
Sergio R. Ambrosio

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Review Article

Application of Traditional Chinese Medical Herbs in Prevention and Treatment of Respiratory Syncytial Virus

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Respiratory syncytial virus (RSV) is a common viral pathogen of the lower respiratory tract, which, in the absence of effective management, causes millions of cases of severe illness per year. Many of these infections develop into fatal pneumonia. In a review of English and Chinese medical literature, recent traditional Chinese medical herb- (TCMH-) based progress in the area of prevention and treatment was identified, and the potential anti-RSV compounds, herbs, and formulas were explored. Traditional Chinese medical herbs have a positive effect on inhibiting viral attachment, inhibiting viral internalization, syncytial formation, alleviation of airway inflammation, and stimulation of interferon secretion and immune system; however, the anti-RSV mechanisms of TCMHs are complicated, which should be further investigated.

1. Introduction

Respiratory syncytial virus (RSV) is classified as a nonsegmented, negative-sense, membrane-bound RNA virus of the family Paramyxoviridae, spread by droplets, and causing repeated airway infections [1]. Respiratory syncytial virus is the most common cause of hospitalization in infants and children with severe acute lower respiratory infections. Additionally, it is the third most common cause of death in children, through the development of fatal pneumonia, after pneumococcal pneumonia and *Haemophilus influenzae* type b infection. It had been estimated that between 66 000 and 199 000 children younger than 5 years died of RSV-associated diseases in 2005, with 99% of these deaths occurring in developing countries [2]. In the 1960s a formalin-inactivated vaccine (FI-RSV) given intramuscularly did not reduce the frequency of infection and among infected children (particularly in the youngest age cohort, immunized between 2 and 7 months of age) 80 percent required hospitalization and 2 died [3–5].

Due to the nature of the virus and the mode of infection, reinfections are a common event, suggesting that naturally acquired immunity does not provide long-lasting protection. This has made the development of an effective vaccine impossible as yet [6]. Currently, the Food and Drug Administration (FDA) has approved prophylactic drugs for RSV, including palivizumab and ribavirin, administered along with symptomatic treatment and supportive care [7]. While palivizumab, a humanized monoclonal antibody directed against the F protein RSV, effectively prevents RSV infection, it is expensive and ineffective in the management of an established RSV infection [8]. Ribavirin has been shown to exhibit potent activity against RSV in vivo and in vitro; however comparisons of the results of both animal and cell testing in humans have not yet been completed [9–11]. Furthermore, ribavirin is limited due to the side effects

[11, 12]. There are any antiviral agents against RSV that are currently under development.

This review aims to explore evidence available on preventative measures against RSV infection, discuss the current status of anti-RSV research on TCMH-derived active compounds, TCMHs, and traditional Chinese medical formulas (TCMFs), and finally highlight the challenges, issues, and future directions of using TCMHs in clinical practice.

2. Evidence Supporting the Efficacy of TCMHs

Traditional Chinese medical herbs are the most important components of the traditional Chinese medicine (TCM) system, which have been reported to cure infectious diseases, in the form of hot water extracts, for almost 2,000 years. Over 10,000 herbal medicines and 100,000 recipes have been documented in ancient literature [13].

Nowadays, TCMHs are widely used for the prevention and treatment of viral infectious diseases in China and many other Asian countries. They are associated with advantages such as lower toxicity, economical and multifunctional use in regulating immunological function, and viral destruction [14]. This reduction in viral load allows for the screening of effective medications. However, the international community remains uncertain about the efficacy of TCMHs, due to the lack of supporting clinical evidence collected under international standards (randomized, placebo-controlled, double-blind, and multicentered clinical studies). Governments have put forward support aimed at the international regulatory approval of TCMHs. Leading the pack is compound T89 (also known as Dantonic, a traditional Chinese medical product by Tasly Pharmaceuticals, China), which is used in China for the management of ischemic heart disease. Dantonic may become the first traditional Chinese medicine to receive Food and Drug Administration (FDA) approval in the United States [15]. Moreover, the Yinhuang granules (tablet) and Xiasangju granules have been approved by the China Food and Drug Administration (SFDA) for the treatment of RSV-infectious diseases [15]. The 2015 Nobel Prize in Medicine was awarded to Youyou Tu for the discovery of artemisinin, a drug isolated from a plant, which has significantly reduced the mortality rates for patients with malaria. This acknowledgement has increased the optimism of the natural product community worldwide [16].

3. Prevention

Prevention is the most important aspect of healthcare, and because of the nature of the respiratory syncytial virus and mode of infection, reinfections are a common event [6]. Live RSV can survive on surfaces for several hours; moreover, direct or indirect contact with the nasopharyngeal secretions or droplets (sneezing, coughing, and kissing), fomites, and food from infected patients can potentially transmit RSV [7]. Therefore, without effective preventive measures, the mortality and economic burden of the disease will increase.

3.1. RSV F Protein Neutralizing Antibodies. The respiratory syncytial virus depends on the host cell to complete its replication cycle, which includes attachment and entry into the host cell, transcription of viral mRNA, viral genome replication, protein synthesis, and the assembly and budding of progeny virus particles [15]. The F protein of RSV accumulates in the host membrane and surrounds the budding progeny viruses, thus spreading the infection to adjacent cells and exacerbating the infection. The F protein is therefore considered a potential candidate for the development of possible preventive measures. Palivizumab is a humanized monoclonal antibody (IgG) directed against an epitope in the A antigenic site of the F protein of RSV. Palivizumab-N, a plant-based F neutralizing antibody, was experimented in phages and plants. Successful palivizumab-N production was observed in the *Nicotiana benthamiana* plant system, which offered glycosylation and high production at low upstream and equivalent downstream cost, when compared to mammalian derived palivizumab [7].

3.2. Boost Resistance against RSV by Modulating Immune Response. Following coevolution with the host, many viruses have established sophisticated mechanisms to interact with the host immune system for immune evasion. Among the classes of antiviral agents, immunomodulators are the most abundant in TCMHs [15]. Traditional Chinese medical herbs have been shown to enhance antibody production, T cell proliferation, the expression of antigen specific CD4+ and CD8+ responses, and increase of the titers of IgG1, IgG2a, and IgA, along with the function to balance Th1, Th2/Th17 type immune response [17]. Interferon- (IFN-) γ and interleukin- (IL-) 12 are representatives of Th1 type cytokines; interleukin 4 and interleukin 5 belong to Th2 type cytokines, and IL-6 and IL-17a are representatives of Th17 type cytokines, all of which play key roles in Th2/Th17 responses [18, 19]. And, recently, macrophages have been identified as stimulation cells by interaction with Th2 cytokine IL-4 [20]. Traditional Chinese medical herbs were reported to simultaneously promote Th1-response, including increased levels of Th1 type cytokine secretion and activation of alveolar macrophages, suppress Th2/Th17-responses, and maintain the balance between Th1 and Th2/Th17 cells for the prevention and treatment of diseases.

Moreover, TCMHs have been previously reported to offer efficient protection against RSV and significantly decrease viral load and inflammation [15]. It is well-known that the production of inflammatory mediators is regulated by a transcription factor, NF-κB, which plays a pivotal role in inflammation due to its ability to induce the transcription of inflammatory genes [21–23]. Toll-like receptor 4 (LR4) is also a factor expressed on the respiratory epithelium and macrophages and appears to play a central role in mediating both the antiviral and inflammatory responses of the innate immunity to combat viral infections [22]. Along with direct antiviral activity, TCMHs could be beneficial in preventing RSV infection due to its immune-modulatory effect, producing of IFN-β and TNF-α. Respiratory syncytial viral infection can induce cellular production of IFN-β and TNF-α. Both IFN and TNF contribute to the innate immunity against viral infection [24] and TNF triggers multiple antiviral

Table 1: Aqueous extracts of TCMHs in prevention of RSV.

Herbs	Used part	Prevention activity	References
Fresh ginger	Rhizome	Showed its better effect when given before viral infection: viral attachment ↓; viral internalization ↓ In HEp-2 cells: IC50 was 212.7 μg/mL (2 h before); in A549 cells: IC50 was 26 μg/mL (2 h before) IC50 was 82.8 μg/mL (1 h before) Secrete IFN-β that contributed to the inhibition of viral replication	[29]
Ginseng (naturally dried)	Root	Anti-inflammatory functions: less weight loss; diminishing pulmonary inflammatory response Inducing host immune responses toward Th1 type immunity: IgG2a isotype antibodies ↑; IFN-γ ↑; IL-4 ↓; CD4 T cell infiltration ↓: cell numbers of CD4+ T (CD3+ CD8−) cells ↓; the ratio of CD8+/CD4+ T cells ↑	[30]
Panax Korean red ginseng (fermented red)	Root	In the prevention of RSV infections: improved survival of human lung epithelial cells against RSV infection; inhibited RSV-induced cellular oxidative damage Play a role in priming the host immune system: enhanced IFN-γ production following RSV viral infection; blocked induction of RSV-induced proinflammatory gene expression in the human alveolar epithelial cell line	[31]
Radix Glycyrrhizae	Rhizome	It is more effective when given before viral inoculation ($P < 0.0001$): inhibition of viral attachment ($P < 0.0001$) and penetration ($P < 0.0001$) In HEp-2 cells: IC50 was 78.0 μg/mL (2 h before) IC50 was 99.7 μg/mL (1 h before); in A549 cells: IC50 was 18.8 μg/mL (2 h before) IC50 was 33.8 μg/mL (1 h before) Stimulate mucosal cells to secrete IFN-β to counteract viral infection	[32]

IL-4: interleukin-4; IFN-γ: interferon-γ; IFN-β: interferon β; Th1 type immunity: T helper type 1 (Th1) immune; A549: human lung carcinoma cell; HEp-2: human larynx epidermoid carcinoma cell.

mechanisms and synergizes with IFN in promoting antiviral activities [25].

Resveratrol, a compound synthesized by at least 72 plant species [26], exhibited certain abilities in the inhibition of RSV replication, inflammation, the level of IL6, virus-induced TIR-domain-containing adapter inducing interferon-β (TRIF), and TANK binding kinase 1 (TBK1) protein expression [27]. Moreover, it has been shown to prevent airway inflammation/hyperresponsiveness during RSV infection in mice, suggesting its applicability in reducing RSV-induced airway symptoms. Additionally, it has also been shown to reduce IFN-γ levels associated with RSV-mediated airway inflammation [28].

In Table 1, we have provided evidences of medicinal herbs used in TCM for the prevention of RSV. This novel approach seems to be a promising and safe prevention option against RSV.

4. Treatment

4.1. TCMH-Derived Active Compounds. Drug use from natural products has progressed to the isolation of active compounds. Natural products and particularly medicinal plants remain an important source of new drugs, new drug leads, and new chemical entities [58, 59]. Drug discovery from nature led to the isolation of early drugs such as quinine, artesunate, and arsenic trioxide, which are active ingredients effectively extracted from natural products and have since proven to be clinically successful. Quinine is made from cinchona bark and artesunate is derived from *Artemisia annua*, and both are used for the treatment of severe malaria [60, 61]. Arsenic trioxide, extracted from white arsenic, is used for the treatment of acute promyelocytic leukemia [62, 63].

Traditional Chinese medical herbs have been utilized as anti-infectious medicines for thousands of years in China and offer various anti-infectious compounds, particularly for antiviral. An increasing number of TCMH-derived active compounds with antiviral activity are garnering evidence of experimental efficacy. As shown in Green Bae's research, (−)-(R)-nyasol, (−)-(R)-4′-O-methylnyasol, and broussonin A, three structurally known compounds isolated from the rhizomes of *A. asphodeloides*, exhibited potent antiviral activities against the RSV-A2 strain propagated in HEp-2 cells. The results showed IC50 values of 0.85, 0.39, and 0.62 μM, respectively, slightly more potent than that of the positive

control ribavirin (IC50 = 1.15 μM) [64]. Table 2 details a list of anti-RSV active compounds derived from TCMHs verified by experiments in vivo or in vitro.

4.1.1. The Characteristics of Active Compounds in TCMHs. A vast amount of basic research has been conducted on TCMHs. Therefore, the isolation and characterization of pharmacologically active compounds from TCMHs and the testing of their pharmacological activities, in the pursuit of new drug discovery, are now necessary [65]. When compared to random screening from a combinatorial chemical library, TCMHs are drugs with a long history of medical use and could be used as the markers for selecting active ingredients from herbal medicines. It would therefore be more efficient to screen active compounds from TCMHs. Furthermore, in biological screening assays, TCMH-derived active compounds often have better pharmacokinetics and bioavailability [15].

Previous studies, as shown in Table 2, have provided evidence of the direct anti-RSV activity of compounds derived from TCMHs. Notwithstanding the failure to discover new chemical structures during drug discovery from medicinal plants, known compounds with new biological activities, such as the inhibition of RSV replication and activation of the immune system, which can provide important drug leads, may be found [66]. Moreover, for countries with limited resources, TCMHs-derived active compounds will serve as a gateway for the merging of modern drug discovery.

However, even with these unique advantages, screening programs focused on identifying potential anti-RSV agents from TCMHs are using conventional methods. In order to compete with other drug discovery methods, efforts are ongoing to replace these traditional methodologies with modern sample preparation and extraction procedures. Therefore, TCMH research needs to continually improve the speed of screening, isolation, and structure elucidation processes. The most challenging issues are understanding the operative mechanisms to identify TCMH-derived active components, as each kind of TCMH contains multiple active components with multiple targets. Some of these components work directly on the therapeutic targets, whereas others may enhance the bioavailability [59]. Therefore, more efforts are needed in the research of compounds in TCMH assays.

It is known that cell-based assays are often initially carried out for the evaluation of whole extracts that show clinical evidence of antiviral activity. Without larger randomized, double-blind, placebo-controlled multicenter clinical trials, approval of TCMH-derived active compounds will be difficult through international regulatory agencies such as the Food and Drug Administration (FDA) of the United States of America or other equivalent European counterparts.

Above all and in long term, TCMHs still offer a great prospect in the field of pharmaceutical developments. The characteristics of TCMHs offer opportunities for finding active and novel chemical structures against a variety of therapeutic targets.

4.2. Traditional Chinese Medical Herbs (TCMHs). Traditional Chinese medicine theory is in line with seasons, circumstances, and individuality. For example, in summer, "*Agastache rugosa*" or "*Eupatorium*" is commonly used in northern China (colder district) to ease the summer-heat and "*ephedra*" and to relieve exterior syndrome ("exterior syndrome" refers to lesions with mild conditions. The symptoms are aversion to cold, fever, headache, body pain, stuffy nose, no sweat, and so on) by diaphoresis while in southern China (warmer district), "honey-fired *ephedra*" is frequently used because of its mild function of stimulating the secretion of sweat. Moreover, the TCM treatment should vary between individuals. For the children with upper respiratory tract infection, the treatment method aims to clear the exterior evil ("evil" is the general name of all kinds of pathogenic factors; "exterior syndrome" discussed above is caused by exterior evil), while, in the elderly, the treatment method aims to strengthen body resistance and relieve the exterior syndrome.

In both 2001 and 2002, approximately one-quarter of the bestselling drugs worldwide were natural products or were derived from natural products [59]. Recent publication shows that TCMHs account for 10% of the prescription drugs in China. They are perceived as harmless and natural and are widely used in several parts of the world individually or in combination [15].

4.2.1. The Characteristics of TCMHs. Traditional Chinese medical herbs are the most important components of the TCM system, which have long been used for the multiple combinations of compounds in the form of processed natural products. Experimental findings of TCMHs with the identification of potent antiviral activities against RSV should be made available to the healthcare providers practicing traditional medicine. From a clinical perspective, diseases are complex and variable, combined with complex clinical symptoms. Traditional Chinese medical herbs contain multiple active components with multiple targets, which may be a better choice to achieve multiacting treatment.

Traditional Chinese medicine theories originate from the profound experiences of practitioners with understanding of ancient Chinese medicine, and the methods of application have been passed down through oral history. It is suggested that hot water extracted TCMHs would exhibit direct antiviral activity. In this review, we summarized experiments in vivo or in vitro (Table 3) that used aqueous extracts of TCMHs for treating RSV-infectious diseases. The objective of these experiments was to review the potential uses of natural products for the treatment of infections caused by RSV.

Ma et al. recently tested 44 medicinal herbs, used in the TCM system, for treating virus-infectious diseases. The medicinal herbs were tested for antiviral activities against RSV using a cytopathologic effect (CPE) assay. Twenty-seven of the 44 medicinal herbs showed moderate or potent antiviral activities against RSV, with a 50% inhibition concentration (IC50) ranging from 6.3 to 52.1 g/mL and a selectivity index (SI) ranging from 2.0 to 32.1. The control group, ribavirin, had an SI of 24 [44].

This evidence illustrates that natural products can be a rich source for the discovery of potent anti-RSV agents.

4.2.2. The Bottleneck Problems of TCMHs. Although traditional natural products have played an important role in

Table 2: TCMHs-derived compounds inhibiting RSV.

(a)

Herbs	Compounds	Anti-RSV activity	References
Folium Isatidis	Fraction III; fraction IV	RSV replication ↓ (SI: 13.06; >24.33; MTT: 11.67; >22.32) The ribavirin group (SI: <28.04; MTT: 40.52)	[33]
Flos Pueraria Omeiensis	Genistein, tectorigenin	Possess potent antiviral activity against RSV (CC50: 450; 500 μg/mL; IC50: 12.5; 30 μg/mL, SI: 36; 16.7); the ribavirin group: (CC50: 62.5 μg/mL; IC50: 3.0 μg/mL; SI: 20.8)	[34]
Radix Gentianae	RG2-1 [35] RG3-1 [36]	RSV replication ↓ (TC50: 10.66; 5.29 mg/mL; EC50: 420; 262.95 μg/mL; TI: 25.38; 20.12) [35, 36]; the ribavirin group (TC50: 1.18; 1.15 mg/mL; EC50: 30.88; 15.48 μg/mL; TI: 38.21; 74.92) [35, 36]	[35, 36]
Herba Patriniae	AP3 [37] AP4 [38]	RSV replication ↓; RSV entry ↓ (TC50: 11.45; 10.89 mg/mL; EC50: 0.0986; 0.0801 mg/mL; TI: 116.12; 135.95) [37, 38]; the ribavirin group (TC50: 0.076, 1.97 mg/mL; EC50: 0.00143, 0.036 mg/mL; TI: 53.45, 54.72) [37, 38]	[37, 38]
Litchi Chinensis	Flavonoids	RSV replication ↓ (TC50: 152.9 mg/mL; IC50: 58.6 mg/mL; TI: 2.6); the ribavirin group (TC50: 154.9 mg/mL; IC50: 57.1 mg/mL; TI: 2.7)	[39]
Radix Glycyrrhizae	18β-GA [32] GD4 [40]	RSV replication ↓ (IC50: 4.3–4.5 μg/mL; CC50: 71.5–76.3 μg/mL; SI: 15.9–17.7) [32]; (EC50: 28.73 μg/mL; TI: 8.0) [40]; the ribavirin group (EC50: 5.67 μg/mL, TI: 215.0) [40]	[32, 40]
Ligustrum lucidum	Oleuropein	Significant antiviral activities against RSV (TC50: 562.5 μg/mL; IC50: 11.7 μg/mL; TI: 48); the ribavirin group (TC50: 62.5 μg/mL; IC50: 2.6 μg/mL; TI: 24)	[41]
Lophatherum gracile	Flavone 6-C-monoglycosides	Potent antiviral activities against RSV (CC50: ranging from 254.5 ± 6.4 to 362.6 ± 15.4 μg/mL; IC50 ranging from 5.7 to 50 μg/mL, SI ranging from 5.6 to 63.6 μg/mL); the ribavirin group (CC50: 62.5 ± 3.2 μg/mL; IC50: 3.0 ± 0.4 μg/mL; SI: 20.8)	[42]
Pithecellobium clypearia	Quercetin	Possess potent antiviral activity against RSV (IC50: 2.5 μg/mL; SI: 180); the ribavirin group (IC50: 3.0 μg/mL; SI: 20.8)	[43]
Sophora flavescens	Anagyrine, oxymatrine, sophoranol	Potent antiviral activities against RSV (IC50: 10.4; 10.4; 10.4 μg/mL; CC50: 250; 125; 250 μg/mL; SI: 24.0; 12; 24); the ribavirin group (IC50: 2.6 μg/mL; CC50: 62.5 μg/mL; SI: 24)	[44]

TC50 is 50% of toxic concentration; EC50 is the concentration for 50% of maximal effect; IC50 is the concentration of the sample required to inhibit virus-induced CPE 50%; CC50 is the concentration of the 50% cytotoxic effect; selective index (SI) or therapeutic index (TI): CC50/IC50 or TC50/EC50 or TC50/IC50; MTT assay is a colorimetric assay for assessing cell metabolic activity.

(b)

Herbs	Compounds	Anti-RSV activity	References
Scutellaria baicalensis	Wgonin oroxylin A	Potent antiviral activities against RSV (IC50: 7.4; 14.5 μg/mL; CC50: 119.2; 58.1 μg/mL; SI: 16.1; 4.0); the ribavirin group (IC50: 2.6 μg/mL; CC50: 62.5 μg/mL; SI: 24)	[44]
Selaginella uncinata	Uncinoside A; uncinoside B	Potently inhibit RSV infection (IC50: 6.9; 1.3 μg/mL; TC50: 82.5; 83.3 μg/mL; TI: 12; 64); the ribavirin group: (IC50: 2.6 μg/mL; CC50: 62.5 μg/mL; TI: 24)	[45]
Wikstroemia indica	Daphnoretin	RSV replication ↓ (IC50: 5.87 μg/mL; SI: 28.17); the ribavirin group (IC50: 3.05 μg/mL; SI: 21.4)	[46]
Radix wikstroemiae	Genkwanol B; genkwanol C; stelleranol	Potent antiviral activities against RSV (IC50: 9.6; 6.6; 10.2 μg/mL; CC50: 106.1; 145.3; 161.5 μg/mL; SI: 11; 21.9; 15.8); the ribavirin group (IC50: 11.9 μg/mL; CC50: 256.1 μg/mL; SI: 21.6)	[47]
Youngia japonica	Fractions 10 [26]; fractions 11 [26]; 3,4-dicaffeoylquinic acid [48]; 3,5-dicaffeoylquinic acid [48]	Syncytium formation of RSV ↓; potent antiviral activities against RSV (IC50: 3.0–6.0 μg/mL; MNCC: 200 μg/mL) [26]; (IC50: 0.5 μg/mL) [48]; the ribavirin group (IC50: 3.0 μg/mL, MNCC: 31 μg/mL) [26]; the ribavirin group (IC50: 2.5 μg/mL) [48]	[26, 48]

TC50 is 50% of toxic concentration; EC50 is the concentration for 50% of maximal effect; IC50 is the concentration of the sample required to inhibit virus-induced CPE 50%; CC50 is the concentration of the 50% cytotoxic effect; selective index (SI) or therapeutic index (TI): CC50/IC50 or TC50/EC50 or TC50/IC50; MNCC: the maximal concentration of the sample that did not exert toxic effect detected by microscopic monitoring after 72 h of incubation.

Table 3: Aqueous extracts of TCMHs inhibiting RSV.

Herbs	Used part	Anti-RSV activity			References
		IC50 (μg/mL)	CC50 (μg/mL)	SI	
Bupleurum chinense	Root	36.8	441.3	12.0	[44]
Gardenia jasminoides		21.0	112.1	5.3	
Isatis indigotica		50.0	1000	20.0	
Ipomoea cairica		52.1	833.3	16.0	
Polygonum multiflorum		52.1	>1000.0	>19.2	
Platycodon grandiflorum		<44.1	352.5	>8.0	
Polygonum cuspidatum Sieb.		<13.0	>200.0	>15.4	
Scutellaria baicalensis		<10.4	>62.5	6.0	
Sophora flavescens		52.1	833.3	16.0	
Tinospora capillipes		17.2	275.8	16.0	
Paeonia suffruticosa	Root cortex	26.0	>833.3	>32.0	
Phragmites communis	Rhizome	46.5	185.8	4.0	
Andrographis paniculata	Aerial parts	27.6	295.0	10.7	
Artemisia capillaries		13.0	208.3	16.0	
Schizonepeta tenuifolia		49.2	196.9	>4.0	
Scutellaria indica		31.3	350.0	11.2	
Perilla frutescens	Leaf	37.6	255.6	6.0	
Lonicera japonica	Flower bud	50.0	>1000.0	>20.0	
Dendranthema morifolium	Flower	50.5	269.2	5.3	
Forsythia suspense	Fruit	50.0	1000.0	20.0	
Prunella vulgaris L.	Fruit spike	<10.4	>62.5	6.0	
Callicarpa nudiflora	Whole plant	37.5	800.0	21.3	[49]
Patrinia villosa		<13.0	416.7	>32.0	
Sarcandra glabra		50.0	1000.0	20.0	
Selaginella sinensis		50.0	>62.5	>20.0	
Blumea laciniata Callicarpa		15.6	243.8	15.5	
Laggera pterodonta		15.6	500.0	31.8	
Mussaenda pubescens		512.5	32.0	16.0	
Schefflera octophylla	Leaf stalk	12.5	500.0	40.0	
Ribavirin [44]	—	2.6	62.5	24.0	[44]
Ribavirin [49]	—	3.0	62.5	20.8	[49]

IC50 is the concentration of the sample required to inhibit virus-induced CPE 50%; CC50 is the concentration of the 50% cytotoxic effect; selective index (SI) or therapeutic index (TI): CC50/IC50.

drug discovery, in the past few years, many pharmaceutical companies have scaled down or terminated their natural product research [59]. Drug discovery programs already follow a challenging process: the design, determination, and implementation of appropriate, clinically relevant, high-throughput bioassays of drugs, and TCMH discovery programs are more complicated and lengthier than other drug discovery methods.

Despite the recognized therapeutic benefits and the expanding use of medicinal plants worldwide, TCMHs still lack robust evidence from evidence-based medicine (EBM) research, especially the supporting clinical evidence collected under international standards (randomized, placebo-controlled, double-blind, and multicentered clinical studies). As a result, it is a challenge for TCMHs to be accepted by the Western medicine community and mainstream healthcare systems.

Though much debate remains on the feasibility and the validity of applying the EBM criteria to this traditional practice, for the integration of TCM into the Western healthcare system, it is essential to demonstrate its efficacy and safety through high level evidence in accordance with EBM procedures.

4.3. Traditional Chinese Medicine Formulas (TCMFs). Based on TCM theory, a remedy often has a principle of hierarchy, the so-called "monarch," "minister," "assistant," and "guide components" [53], and the functions differ among the different roles. The basic principle of TCM treatment is to keep the balance between Yin and Yang (two opposite as well as relevant agents, the main components of the universe in Chinese

philosophy) and Qi and blood (two basic components of the human body, with the functions of maintaining the life).

A TCM formula contains multiple active herbs with multiple compounds and targets. Some of these components ("monarch" and "minister") work directly on the therapeutic targets, while others ("assistant" and "guide components") enhance the bioavailability or counteract drug toxicity of the medicine [15]. This review summarized some TCMFs and Chinese patent medicines in Tables 4 and 5, which had anti-RSV activities, supporting their possible use in managing RSV infection.

4.3.1. The Characteristics of TCMFs and Chinese Patent Medicines. Traditional Chinese Medicine Formulas have been used as remedies against infectious diseases for thousands of years due to their significant anti-inflammatory, antimicrobial, and antiviral activities, with little or no adverse effects [44, 67]. Therapeutic effects of mixed formulas are based on the synergic action of its mass constituents [67]. Currently, Chinese patent medicines, with convenient dosage forms, represent the majority of drugs on the market. Anti-RSV Chinese patent medicines (during infection) are usually considered "cold" herbs and are applied to remove the heat-evil ("heat evil" related to the clinical manifestations characterized by ulcers local swelling and heat pain), expel superficial evils (superficial evils lead to "exterior syndrome," which is explained in previous 17th paragraph), and dispel toxins from the body, such as Shuang Huang Lian oral liquid, Jin Xin oral liquid, Qing Fei oral liquid, or Xiasangju granules (Table 4). The Xia Sang Ju granule was approved by the China Food and Drug Administration (SFDA), and Jin Xin oral liquid, a traditional Chinese patent medicine, has been widely used for decades to treat virus-infectious pneumonia in the Jiangsu Province Hospital of Nanjing University of Traditional Chinese Medicine, Nanjing, China. Following recovery from an infection, Bu Shen Yi Qi formula is administered, which is a traditional Chinese herbal formula composed of herbs used to tonify Qi of the kidney, that is, the strengthening of the body's resistance to eliminate pathogenic factors. The anti-RSV mechanism of Bu Shen Yi Qi formula was investigated in the Th1, Th2, and Th17 cells [50].

Based on the improvements in experiments in vivo or in vitro, a number of Chinese patent medicines are currently undergoing FDA clinical trials in the hope of fulfilling EBM research criteria. This includes Danshen dripping pill (*Salvia miltiorrhiza*, *Radix notoginseng*, and Borneol) for stable angina in a phase III trial, Fu zheng Hua yu capsule (*Salvia miltiorrhiza*, peach seed, pine pollen, *Gynostemma pentaphylla*, and *Schisandra chinensis*) for liver fibrosis, Kang lai te injection (coix seed oil and excipients) for cancer, and the Xue zhi kang capsule (red yeast) for hyperlipidemia in phase II trials [71].

Traditional Chinese Medicine Formulas rely on written traditional Chinese medicine and an educational system of TCM scholars. According to Shanghan Lun (Treatise on Cold Pathogenic Diseases), written by Zhang Zhongjing, TCMFs should be administered three times. A dosage three times a day is recommended to avoid an overdose and to ensure the better absorption of drugs. This is to ensure a balanced blood drug concentration, according to modern medicine [72].

In TCM theory, the quality of the decoction is the key to cure diseases successfully; hence detailed requirements of the decocting methods should be illustrated. The methods of decocting medicinal herbs were passed down through the form of hot water extracts [44]; the oral form of the decoction would exhibit major effects, such as direct antiviral activity. The decocting time differs from herbs to herbs. For aromatic plants, such as *Mentha* and *Agastache rugosa*, the drug efficacy will be lost with lengthened decocting; therefore these plants should be decocted for only 5–10 minutes, while for plants with a certain level of toxicity, such as *Aconitum carmichaelii* and *Pinellia ternata*, decocting should be at least 1 hour to reduce its toxicity.

Compared to Western medicines, advantages of TCMFs include individual treatment based on "multicomponents, multichannels, and multitargets" application and syndrome differentiation. The characteristics of TCMFs are to balance the body and improve clinical symptoms of a patient. Considering these unique characteristics, development of TCMFs, in Tables 4 and 5, with anti-RSV activity is of great importance. However, this clinical application needs further clinical validation.

4.3.2. The Bottleneck Problems of TCM Formulas. With the development of science and technology and the increasing need of modern clinical practice, the disadvantages of oral TCM preparations have gradually been realized. These include single administration, old formulation, large dosage, slow effect, and outdated preparation and transport methods.

The development of Chinese patent medicines has aided the classification; however, in treating acute and severe diseases, TCM formulas are criticized for their slow effect and large dosage. This prevents TCM from being able to solve urgent medical diagnoses. Moreover, dosage needs to be improved: for a convenient use of the drug and the improvement of the therapeutic effect, the dosage needs to be improved while upholding the active effect. More experiments and clinical trials are needed in the field of TCMFs.

5. Future Directions in the Prevention and Treatment of RSV

5.1. Attachment/Internalization Inhibitors. The spread of RSV is initiated through viral attachment and internalization. The severity of the RSV-infectious disease is positively correlated with the viral load [44]; therefore, inhibition of viral spread between airway mucosal cells to minimize viral replication and the decrease viral load is essential for disease control.

Traditional Chinese medical herbs can prevent the respiratory viral infection induced by RSV, by inhibiting viral attachment and penetration and reducing the increased susceptibility of the cell to the invasion of RSV. From details in Table 2 to Table 5, conclusions can be made that TCMHs can effectively inhibit viral attachment and cell internalization, in order to minimize the viral spread and replication.

Table 4: Chinese patent medicine in prevention and inhibition of RSV.

(a)

Names	Plant or mineral	Composition Weight (g) (%, w/w)	Used part	Mechanisms and results	References
Bu Shen Yi Qi Tang (BSYQF) (granules)	*Radix Astragali*	46.15%	Root	Airway inflammation ↓; RSV replication ↓; F protein expression of RSV ↓ ($P < 0.05$) Regulating the balance between Th1 and Th2/Th17 responses: Th1-response ↑: CD4+ T cell proportion ↑; T-Bet ↑; macrophages ↑; IFN-γ ↑; IL-12 ↑ Th2/Th17 cell proportions ↓: GATA3 ↓; STAT6 ↓; RORγT ↓; IL-4 ↓; IL-5 ↓; IL-6 ↓; IL-17a ↓	[50]
	Herba Epimedii; Rehmanniae Radix	30.77% 23.08%	Aerial part, root tuber		
Shuang Huang Lian oral liquid (SHL)	*Scutellariae baicalensis Georgi*	25.00%	Rootstalk	RSV replication ↓; the virus titer ↓; airway inflammation ↓; IL- 8 ↓; the SHL group: TC50 > 160 μg/mL; the ribavirin group was similar to the SHL group ($P > 0.05$); no adverse reaction was found	[51, 52]
	Lonicera japonica	25.00%	Flower bud		
	Forsythia suspensa	50.00%	Fruit		
Sheng Ma Ge Gen Tang (SMGGT)	*Pueraria lobata Ohwi*	33.33%	Radix	The crude extract of SMGGT was more effective when given before viral infection ($P < 0.0001$): viral attachment ↓; RSV internalization ↓ The SMGGT group: CC50: 3000 μg/mL; IC50: 34.2–82.8 μg/mL; the ribavirin group was similar to the SMGGT group ($P > 0.05$) Epithelial cells secrete IFN-β ↑ and TNF-α ↑ beforehand and after viral inoculation to defense RSV	[24]
	Paeonia lactiflora Pallas	22.22%	Radix		
	Cimicifuga foetida L.	22.22%	Rhizoma		
	Glycyrrhiza uralensis Fischer et DC	11.11%	Radix		
	Zingiber officinale Roscoe	11.11%	Root-like stem		
Xia Sang Ju Granule (XSJG)	*Prunella vulgaris L.*	66.23%	Fruit spike	RSV replication ↓; the virus titer ↓; the XSJG group: TC50: 800 μg/mL; IC50: 544.59 μg/mL; TI: 1.47; the ribavirin group was similar to the XSJG group ($P > 0.05$); no adverse reaction was found	[53]
	Dendranthema indicum	23.18%	Flower		
	Mulberry leaf	10.60%	Leaf		

Th1 cell: T helper 1 cell; Th2 cell: T helper 2 cell; Th17-cell: T helper 17 cell; GATA3: transcription factors GATA binding protein 3; STAT6: signal transducer and activator of transcription 6; RORγT: retinoid-related orphan receptor gamma t; T-Bet: T box expressed in T cells; IL: interleukin; A549: human lung carcinoma cell; TC50 is 50% of toxic concentration; IC50 is the concentration of the sample required to inhibit virus-induced CPE 50%; CC50 is the concentration of the 50% cytotoxic effect; IFN-β: interferon β; IFN-γ: interferon-γ; TNF-α: tumor necrosis factor-α.

(b)

Names	Plant or mineral	Composition Weight (g)	Used part	Mechanisms and results	References
Jin Xin oral liquid (JOL)	*Ephedra sinica Stapf*	3	Stem	Lung inflammation ↓; RSV replication ↓; the viral load in the lung tissues ↓	[54]
	Prunus armeniaca L.	10	Seed		
	Morus alba L.	10	Velamen		
	Scutellariae baicalensis Georgi	10	Rootstalk	During early stage infection (on days 2 and 4 after infection), the TLR3-IRF3-IFN-β signaling pathway ↓: IFN-β ↓; TLR3 ↓; IRF3 (p-IRF3) ↓; the expression of SOCS1 ↓	
	Peucedanum praeruptorum Dunn	10	Root		
	Lepidium apetalum Willd.	10	Seed	During the later stage of infection (7 d after infection), the TLR3-IRF3-IFN-β signaling pathway ↑: IFN-β ↑; TLR3 ↑; IRF3 (p-IRF3) ↑; the expression of SOCS1 ↓	
	Polygonum cuspidatum Sieb.	10	Root		
	Gypsum ($CaSO_4 \cdot 2H_2O$)	15	—		

(b) Continued.

Names	Plant or mineral	Composition Weight (g)	Used part	Mechanisms and results	References
Qing Fei oral liquid (QOL)	*Ephedra sinica Stapf*	4	Stem	A multicentered single-blind, random controlled clinical study of 166 children in Nanjing in China showed that the curative and the effective rate of the QOL group: 80.00%; 19.38%; the ribavirin group: 41.14%; 41.14%; no adverse reaction was found	[55]
	Prunus armeniaca L.	10	Seed		
	Morus alba L.	10	Velamen		
	Bombyx mori Linnaeus	6	Bombycidae		
	Peucedanum praeruptorum Dunn	10	Root		
	Lepidium apetalum Willd.	6	Seed	Airway inflammation ↓; IL-6 ↓; IL-8 ↓	[56]
	Polygonum cuspidatum Sieb.	12	Root		
	Gypsum ($CaSO_4 \cdot 2H_2O$)	24	—	RSV replication ↓; regulating immunabitity INF-γ ↑	[57]
	Salvia miltiorrhiza Bge	6	Rootstalk		
	Polygonum bistorta L.	12	Rootstalk		

TLR3: toll-like receptors 3; IRF3: interferon regulatory factor 3; IFN-β: interferon β; SOCS1: suppressor of cytokine signaling 1; IL-6: interleukin-6; IL-8: interleukin-8; IFN-γ: interferon-γ; A549: human lung carcinoma cell; TC50 is 50% of toxic concentration; IC50 is the concentration of the sample required to inhibit virus-induced CPE 50%; therapeutic index (TI): TC50/IC50.

5.2. Enhancement of Immunity. Traditional Chinese medical herbs can counteract RSV infection not only through the direct inhibition of viral attachment, internalization, or replication, but also by the enhancement of immunity (Table 6). They are considered to be less toxic and may warrant further evaluation as a possible agent for RSV prevention.

Antiviral cytokines play an important role in the immune system process of preventing and inhibiting RSV infection. Traditional Chinese medical herbs can stimulate antiviral cytokines such as IFN-β and TNF-α, before and after RSV inoculation. TCMHs have also been shown to keep the balance between Th1 and Th2/Th17 immune response, enhance antibody production and T cell proliferation, enhance the expression of antigen specific CD4+ and CD8+ responses, and increase the titers of IgG1, IgG2a, and IgA [17].

5.3. Future Directions. Despite the evident successes of drug discovery from medicinal plants, future endeavors face many challenges. Efforts are needed to continuously improve the quality and quantity of TCMH-derived compounds, herbs, and formulas that enter the drug development phase, in an effort to compete with other drug discovery efforts [59]. The future of current TCMH research is to meet international standards, including evidence-based efficacy (particularly through multicenter, randomized, double-blind, placebo-controlled clinical trials), safety assessment, and quality and quantity control. The aims for the future are that large TCM-focused databases become available and innovative strategies are applied to improve the process of plant collection. Moreover, it is necessary to establish a standardized and centralized research system, aimed at achieving a better understanding of medicinal chemistry and the mechanism of action of TCHMs. The challenges associated with anti-RSV herbs are many. However, at the current pace of scientific research and development, traditional Chinese medical herbs can play a role in effective prevention and treatment of RSV-infectious diseases.

6. Conclusions

Prevention is the most important aspect of healthcare; however due to the nature of the RSV and its mode of infection, a safe and effective RSV vaccine has remained elusive. Traditional Chinese medical herbs can enhance immunity to prevent RSV infection, and, furthermore, TCMHs can form part of anti-RSV medications by inhibiting viral attachment, viral penetration, and reducing the increased susceptibility of the cell to the invasion of RSV. This review details evidence that TCMH can be effective as preventative or treatment medications for RSV, the advantages of lower cost, better patient outcomes, and fewer adverse reactions. From the research that has been conducted in this review, we can see great potential value of conducting trials using TCMH. However, further research, especially focused on the toxicity issues, is required to develop the level of evidence to support TCMH to be applied to practice.

Competing Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Table 5: TCM formulas in prevention and inhibition of RSV.

(a)

Names	Composition			Mechanisms and results	References
	Plant or mineral	Weight (g)	Used part		
Modified Ding Chuan Tang (MDD)	*Salviae miltiorrhizae Radix*	12	Root	MDD exhibited significant antiviral and anti-inflammatory effects: lung viral loads ↓; the MDD group: IC50: 27.2 μg/mL; CC50: >1000 μg/mL; SI: >36.8; the ribavirin group: IC50: 2.8 μg/mL; CC50: 41.6 μg/mL; SI: 14.9 eotaxin ↓; IL-4 ↓; IFN-γ ↓ in serum and lung tissue; TLR4 ↓; NF-κB ↓ in the lung tissue	[67]
	Scutellariae Radix	9	Root		
	Farfarae Flos	10	Flower		
	Ephedra sinica Stapf	4	Stem		
Ge Gen Tang (GGT)	*Cinnamomum cassia Blume*	3.0	Twig	GGT was more effective given before than after viral inoculation ($P < 0.0001$); viral attachment ↓; viral internalization ↓; in A549 cells: IC50 was 8.8 μg/mL (2 h before); IC50 was 27.1 μg/mL (1 h before); IC50 was 184.7 μg/mL (1 h after); the immune-modulatory effect: IFN-β ↑; GGT will not increase TNF-α secretion, but rather inhibit TNF-α secretion in HEp-2 cells	[1]
	Ephedra sinica Stapf	4.5	Stem		
	Glycyrrhiza uralensis Fischer et DC	3.0	Radix		
	Paeonia lactiflora Pallas	3.0	Radix		
	Pueraria lobata Ohwi	6.0	Radix		
	Zingiber officinale Roscoe	4.5	Root-like stem		
	Ziziphus jujuba Mill. var. inermis (Bge.) Rehd.	4.0	Fruit		
Jia Wei Yu Ping Feng Tang (JYT)	*Radix Astragali*	27.7	Root	Viral attachment ↓; RSV internalization ↓; lung lesions and complication ↓; inhibiting the expression of ICAM1; in A549 cells: the JYT group: CC50: 1373.37 ± 2.8 μg/mL, IC50: 97.0 μg/mL; the ribavirin group: CC50: 579.47 ± 7.4 μg/mL; the IC50 value of ribavirin was similar; JYD could inhibit RSV infection in all three modes of treatment but was more effective in the simultaneous and posttreatment modes ($P < 0.05$)	[68]
	Rhizoma Atractylodis Macrocephalae	16.7	Rhizome		
	Radix Saposhnikoviae	16.7	Root		
	Flos Lonicerae japonicae	16.7	Flower bud		
	Rhizoma Dryopteris Crassirhizoma	11.1	Rhizome		
	Pericarpium Citri Reticulatae	11.1	Fruit		

IC50 is the concentration of the sample required to inhibit virus-induced CPE 50%; CC50 is the concentration of the 50% cytotoxic effect; selective index (SI) or therapeutic index (TI): CC50/IC50; NF-κB: nuclear factor-kappa B; IL: interleukin; IFN-γ: interferon-γ; IFN-β: interferon β; TLR4 inhibitor: toll-like receptor 4; A549: human lung carcinoma cell; HEp-2: human larynx epidermoid carcinoma cell; ICAM-1: intercellular cell adhesion molecule-1.

(b)

Names	Composition			Mechanisms and results	References
	Plant or mineral	Weight (g)	Used part		
Liu He Tang (LHT)	*Agastache rugosa*	2.0	Whole plant	LHT was more effective when given before viral inoculation ($P < 0.0001$); viral attachment ($P < 0.0001$) ↓; RSV internalization ($P < 0.0001$) ↓; CC50: 3000 μg/mL; IC50: 34.2–82.8 μg/mL; IC50 value of ribavirin was similar; LHT stimulated epithelial cells to secrete IFN-β and TNF-α to counteract HRSV infection before infection becomes established	[69]
	Amomum villosum Lour.	1.5	seed		
	Atractylodes macrocephala Koidz.	2.0	Root-like stem		
	Chaenomeles sinensis	2.0	Fruit		
	Dolichos lablab L.	2.0	Seed		
	Glycyrrhiza uralensis Fischer et DC	1.5	Radix		
	Magnolia officinalis Rehd. et Wils.	2.0	Bark		
	Panax ginseng C.A. Meyer	1.5	Root		
	Pinellia ternata (Thunb.) Breit.	1.5	Root and stem		
	Poria cocos (Schw.) Wolf	2.5	Sclerotium		
	Prunus armeniaca L.	1.5	Seed		
	Zingiber officinale Roscoe	1.5	Root-like stem		
	Ziziphus jujuba Mill. Var. inermis (Bge.) Rehd.	1.5	Fruit		

(b) Continued.

Names	Composition			Mechanisms and results	References
	Plant or mineral	Weight (g)	Used part		
Xiao Qing Long Tang (XQLT)	*Ephedra sinica Stapf*	9.0	Stem	Hot water extract of XQLT dose-dependently inhibited HRSV-induced plaque formation when given before viral inoculation ($P < 0.0001$); viral attachment ↓ ($P < 0.0001$); RSV internalization ↓ ($P < 0.0001$); CC50: 300 μg/mL; IC50: 34.2–82.8 μg/mL; IC50 value of ribavirin was similar; in A549 cells: IC50 was 22.6 μg/mL (2 h before); IC50 was 50.4 μg/mL (1 h before); IC50 was 128.1 μg/mL (1 h after); secreting IFN-β ↑ before and after viral inoculation to counteract viral infection ($P < 0.0001$)	[70]
	Cinnamomum cassia Blume	6.0	Twig		
	Paeonia lactiflora Pall.	9.0	Root		
	Glycyrrhiza uralensis Fisch.	6.0	Root		
	Zingiber officinale Rosc.	3.0	Rhizome		
	Pinellia ternata (Thunb.) Breit.	9.0	Tuber		
	Asarum heterotropides F Maekawa	3.0	Whole plant		
	Schisandra chinensis (Turcz.) Baill.	3.0	Fruit		

IC50 is the concentration of the sample required to inhibit virus-induced CPE 50%; CC50 is the concentration of the 50% cytotoxic effect; selective index (SI) or therapeutic index (TI): CC50/IC50; IFN-β: interferon β; TNF-α: tumor necrosis factor-α; A549: human lung carcinoma cell.

Table 6: The mechanisms of TCMHs-based prevention and treatment of RSV.

Prevention	Treatment
Viral attachment ↓ [1, 24, 29, 32, 68–70]; viral internalization ↓ [1, 24, 29, 32, 68–70]	RSV entry ↓ [24, 32–49]
IFN-β, TNF-α production → viral replication ↓ [1, 24, 29, 32, 54, 69, 70]	RSV replication ↓ [32–49, 51–55, 57]
IFN-γ production → RSV viral infection ↓ [30, 31, 50, 57, 67]	Syncytium formation of RSV ↓ [26]
The survival of human lung epithelial cells ↑ [31]	Lung inflammation ↓ [30, 50–52, 54–56, 67, 68]
Activate the immune system [30, 50, 57]	F protein expression of RSV ↓ [50]
Blocked proinflammatory gene expression [31, 54, 68] (in the human alveolar epithelial cell line)	Viral loads in serum and lung tissue ↓ [51–54, 67]; no adverse reaction was found [53, 55]

Authors' Contributions

All the authors wrote the first draft of the paper together. All the authors contributed to the review of all papers and constructed the final report.

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Research Article

Tanreqing Injection Attenuates Lipopolysaccharide-Induced Airway Inflammation through MAPK/NF-κB Signaling Pathways in Rats Model

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Background. Tanreqing injection (TRQ) is a commonly used herbal patent medicine for treating inflammatory airway diseases in view of its outstanding anti-inflammatory properties. In this study, we explored the signaling pathways involved in contributions of TRQ to LPS-induced airway inflammation in rats. *Methods/Design*. Adult male Sprague Dawley (SD) rats randomly divided into different groups received intratracheal instillation of LPS and/or intraperitoneal injection of TRQ. Bronchoalveolar Lavage Fluid (BALF) and lung samples were collected at 24 h, 48 h, and 96 h after TRQ administration. Protein and mRNA levels of tumor necrosis factor- (TNF-) α, Interleukin- (IL-) 1β, IL-6, and IL-8 in BALF and lung homogenate were observed by ELISA and real-time PCR, respectively. Lung sections were stained for p38 MAPK and NF-κB detection by immunohistochemistry. Phospho-p38 MAPK, phosphor-extracellular signal-regulated kinases ERK1/2, phospho-SAPK/JNK, phospho-NF-κB p65, phospho-IKKα/β, and phospho-IκB-α were measured by western blot analysis. *Results*. The results showed that TRQ significantly counteracted LPS-stimulated release of TNF-α, IL-1β, IL-6, and IL-8, attenuated cells influx in BALF, mitigated mucus hypersecretion, suppressed phosphorylation of NF-κB p65, IκB-α, IKKα/β, ERK1/2, JNK, and p38 MAPK, and inhibited p38 MAPK and NF-κB p65 expression in rat lungs. *Conclusions*. Results of the current research indicate that TRQ possesses potent exhibitory effects in LPS-induced airway inflammation by, at least partially, suppressing the MAPKs and NF-κB signaling pathways, in a general dose-dependent manner.

1. Introduction

Tanreqing injection (TRQ) is a widely used classical compound herbal recipe for several decades in China. It is composed of water soluble natural extractives from five crude herbal plants, namely, Radix Scutellariae Baicalensis, Fel Selenarcti, Cornu Naemorhedi, Flos Lonicerae, and Forsythiae Fructus [1], and is a mixture of about 12 main active pharmaceutical ingredients including *chlorogenic acid*, *caffeic acid*, *luteoloside*, *forsythiaside*, *forsythin*, *forsythigenol*, *baicalin*, *wogonoside*, *wogonin*, *salidroside*, and *ursodeoxycholic acid* [2]. Clinical evidence has supported the minimal toxicity and side effects of TRQ [3, 4]. With its predominant antibacterial and antiviral actions being proved by modern pharmacologic studies [5, 6], TRQ is predominately used for acute inflammatory lung diseases including acute upper respiratory tract infections [7], pneumonia [3, 8, 9], acute COPD [4, 10, 11], SARS (Serious Acute Respiration Symptom) [12, 13], A/H1N1 flu [14, 15], A/H7N9 flu [16], and the recently mentioned Middle East Respiratory Syndrome (National Health and Family Planning Commission of China, http://www.nhfpc.gov.cn/).

Stimulation of MAPKs and IKK/IκB/NF-κB pathways is common phenomena in many types of inflammation response. Targeting the pathways has been considered as an effective therapeutic approach to mitigate progression of numbers of inflammatory disorders, such as the classical neutrophil-predominant airway inflammation induced by Lipopolysaccharide (LPS) [17]. The involvement of the ubiquitous nuclear transcription factor NF-κB in the pathogenesis of the LPS-induced inflammatory response has been well recognized [18]. LPS liberates and activates NF-κB mostly through IκB kinase- (IKK-) dependent phosphorylation and

subsequent degradation of IκB-α [19]. The liberated NF-κB dimers are crucial for regulating transcription of diverse genes coding for cytokines including TNF-α, IL-1β, IL-6, and IL-8 [20, 21], which together contribute to the upregulation of inflammatory responses. Similar to IKK/IκB-α/NF-κB pathway, the mitogen-activated protein kinase (MAPK) pathway is also activated during an LPS-challenged inflammatory condition [22]. The three subfamilies, extracellular regulated kinases (ERK1/2), c-Jun N-terminal kinases (JNK), and p38 MAP kinases, have been implicated in the release of proinflammatory cytokines [23, 24] and the activation of NF-κB [25].

Prior studies have given consistent results on the ability of TRQ to alleviate LPS-induced inflammation through multiple actions including scavenging excessive oxygen free radicals, suppressing the activation and expression of NF-κB, decreasing serum NO level, and downregulating the expression of Caspase-3 and Bcl-2/Fas gene [12, 13, 26–28]. Collectively, no efforts have been done to understand the pathways that are involved in its effect. Since MAPKs and NF-κB pathway has been highlighted during the LPS-induced inflammatory disorders, we hypothesize that protective actions of TRQ are likely, at least partially, due to its regulations on the two pathways. In the present study, we attempted to elucidate the anti-inflammatory potential of TRQ on LPS-induced airway inflammation in rat models by investigating the pivotal molecular basis involved in the two classical signaling pathways.

2. Material and Methods

2.1. Animals and Drugs. 75 male specific-pathogen-free (SPF) grade SD rats (10–12 weeks old, initially weighing 180–220 g) were raised under SPF conditions on a 12 h light/dark cycle at a temperature of 22 ± 2°C with free access to food and water. Protocol of this experiment was approved by the Animal Care and Use Committee of West China Hospital and the investigation conformed to the "Guide for the Care and Use of Laboratory Animals" [29]. SD rats used for the experiment were obtained from Jianyang Animal and Science Co., Ltd. (Sichuan, China). Tanreqing injection was purchased from Shanghai Kai Bao Pharmaceutical Co., Ltd. (Shanghai, China).

2.2. Experimental Groups and Protocol. Rats were randomly divided into four groups (n = 15 per group): (a) control group: rats receiving sterile saline; (b) LPS group: rats receiving intratracheal instillation (i.t.) of LPS + intraperitoneal injection (i.p.) of sterile saline; (c) low dose TRQ group: rats receiving LPS i.t. + 2.8 mL/kg TRQ i.p.; and (d) high dose TRQ group: rats receiving LPS i.t. + 5.6 mL/kg TRQ i.p. LPS (Sigma, St. Louis, MO, USA) was administered intratracheally during inspiration once at baseline, at a dose of 240 μg/rat. TRQ would be injected 1 h before LPS administration at baseline and then every 24 hours.

2.3. Bronchoalveolar Lavage Fluid (BALF) and Tissue Extraction. On 24 h, 48 h, and 96 h, which have been previously reported as optimal time points to observe the anti-inflammatory activity of TRQ [28], rats were intraperitoneally anaesthetised with 4% sodium pentobarbital (40 mg/kg) and then exsanguinated from the abdominal aorta. The chest cavity was opened by a midline incision to expose and cannulate the trachea. The right lobe of the lung was ligated at the hilus of the lung, and the left lung was immediately lavaged three times with 2 mL ice sterile saline through the tracheostomy tube. Fluid recovery was always above 90% of the original volume. Pooled BALF samples were centrifuged and the supernatants were collected and stored at −80°C for cytokine ELISA. The deposited cells were resuspended for total leukocyte count and the differential leukocyte classification, counting 500 cells from each rat. An experienced investigator who was independent of experimental operations did all the enumerations based on standard morphological criteria. The right middle lobe of the lung was preserved in 4% paraformaldehyde for 24 h at 4°C for histology and histochemical studies. The right posterior lobe of the lung was snap preserved in liquid nitrogen and stored at −80°C for mRNA and protein analysis.

2.4. Enzyme-Linked Immunosorbent Assay (ELISA). Quantitation of cytokines in the supernatants of undiluted BALF and lung homogenate was determined by ELISA technique, according to the manufacturer's instructions. Three replicates were carried out for each of the different treatments. Rat IL-1β ELISA kit was purchased from Shanghai ExCell Biology Company (Shanghai, China) (Cat. Number: ER008-96; Sensitivity: 15 pg/mL; Assay Range: 31.25~2000 pg/mL). Rat CXCL-1/CINC-1 ELISA kit (rat analogue of human IL-8) (Cat. Number: RCN100; Sensitivity: 1.3 pg/mL; Assay Range: 7.8~500 pg/mL), Rat IL-6 ELISA kit (Cat. Number: R6000B; Sensitivity: 0.7 pg/mL; Assay Range: 3.1~700 pg/mL), and Rat TNF-α ELISA kit (Cat. Number: RTA00; Sensitivity: 5 pg/mL; Assay Range: 12.5~800 pg/mL) were purchased from USA R&D Systems (Minneapolis, MN, USA). Samples were applied to wells of 96-well polystyrene microtiter plates that were precoated with specific monoclonal antibodies before incubation, and then wells were washed five times, followed by incubation with the respective HRP-conjugated polyclonal antibodies. After the repeat of aspiration and washing steps, the substrate solutions and stop solutions were added one after another. The optical density of each well was determined at 450 nm using a microplate reader (Bio-Rad, Richmond, CA) within 30 minutes.

2.5. Histopathology and Immunohistochemistry. Fixed specimen was rinsed in PBS, dehydrated, and embedded in paraffin according to standard procedures and serially sectioned at 4 micrometer. Then sections were stained with haematoxylin and eosin (H&E) and Alcian blue (AB)/periodic acid-Schiff (PAS) for general morphology evaluation, which were subsequently practiced by a pathologist who was blinded to group allocation under a light microscopy. The evaluation of inflammation lesions was performed using a subjective numeric scale ranging from 0 to 10, which comprises three scoring parts including peribronchial/peribronchiolar

inflammation score, perivascular inflammation score, and alveolar inflammation score. Peribronchial/peribronchiolar and perivascular inflammations were individually scored from 0 to 4, representing normal (score 0), mild inflammation (score 1, <25%), moderate inflammation (score 2, 25–50%), severe inflammation (score 3, 50–75%), and very severe inflammation (score 4, >75%), respectively [30]. Alveolar inflammation was scored from 0 to 2 that represents normal (score 0), mild inflammation infiltration (score 1, few foci present), and severe inflammation infiltration (score 2, many foci present). The scores were then summed to give a total inflammatory score. Percentage of AB/PAS positively stained areas to the total area of bronchial epithelium was measured.

For p38 MAPK and NF-κB immunohistochemical staining, paraffin-embedded sections were deparaffinized, rehydrated, and washed with distilled water. The monoclonal antibodies used were as follows: rabbit anti-p38 MAPK (1 : 400 dilution) and rabbit NF-κB p65 (1 : 800 dilution). Both antibodies were purchased from Cell Signaling (Danvers, MA, USA). PBS was used to replace the primary antibody as a blank control. The mean optical density (IOD) was calculated by measuring 10 consecutive visual fields for each sample at a magnification of 400x, using an optical microscope equipped with an Image-Pro Plus software (version 6.0, Media Cybernetics, Silver Spring, MD, USA) by a pathologist who was blinded to the identity of the groups.

2.6. RNA Extraction, Reverse Transcription, and Quantitative Real-time Polymerase Chain Reaction. Frozen tissue was ground to a fine powder in liquid nitrogen. After the samples were thawed, total RNA was isolated from 30 mg lung tissue using the E.Z.N.ATM HP total RNA kit (Omega Biotech, Norcross, GA, USA) according to specific modifications to maximize RNA extraction. RNA pellets were ethanol-precipitated, washed, and resuspended in sterile ribonuclease-free water. RNA samples were reverse transcribed using the iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA, USA) to synthesize the first-strand cDNA. Reverse transcription was then performed on 1 μL RNA sample by adding iScript reagents to a final reaction volume of 20 μL. The RNA samples were incubated in a Bio-Rad DNA Engine Peltier Thermal Cycler (Bio-Rad Laboratories, Inc., Hercules, CA, USA) at 25°C for 5 min and then reverse transcribed at 42°C for 30 min and, finally, the enzyme was denatured at 85°C for 5 min. Subsequently, the RNA concentrations were determined by measuring the absorbance at 260 and 280 nm, using a Nanodrop spectrophotometer (Montchanin, DE, USA).

PCR primers specific for selected target genes were predesigned and validated (Table 1). Gradient PCRs were used to determine the optimal annealing temperature and primer concentration. qPCR reactions had a final volume of 10 μL and contained 1 μL of cDNA, 0.5 μL of each primer, 5 μL SsoFast EvaGreen supermix (Bio-Rad Laboratories, Hercules, CA, USA), and 3 μL DEPC-treated sterile distilled water. The relative expression levels of mRNA of studied cytokines were calculated relative to β-actin. Each qPCR was performed in triplicate for the individual sample. The PCR program was initiated by a 30 s of enzyme activation at 95°C and then 5 s of cDNA denaturation at 95°C, followed by 40 cycles at 55°C for 20 s of annealing/extension using Roche LightCycler® 96 Real-Time PCR System. A melting-point curve was then measured, starting from 65°C and increasing by 4.4°C every second up to 95°C, to detect any nonspecific PCR products. Data was analysed using $2^{-\Delta\Delta Ct}$ method with actin as the reference gene.

Table 1: The primer sequences.

TNF-α-forward	TGCTATCTCATACCAGGAGA
TNF-α-reverse	GACTCCGCAAAGTCTAAGTA
IL-6-forward	TCTTGGGACTGATGTTGTTG
IL-6-reverse	TAAGCCTCCGACTTGTGAA
IL-1β-forward	GCAACTGTTCCTGAACTCAACT
IL-1β-reverse	ATCTTTTGGGGTCCGTCAACT
CXCL-1/CINC-1-forward	CTCCAGCCACACTCCAACAGA
CXCL-1/CINC-1-reverse	CACCCTAACACAAAACACGAT
β-actin-forward	CCT CAT GAA GAT CCT GAC CG
β-actin-reverse	ACC GCT CAT TGC CGA TAG TG

2.7. Western Blot Analysis. Nuclear and cytosolic extracts were prepared using a Nuclear and Cytoplasmic Protein Extraction Kit (Beyotime Co., Jiangsu, China) according to the manufacturer's instructions. The concentration of the protein was measured using a bicinchoninic acid protein assay kit (Beyotime Co., Shanghai, China). A total of 50 ug of protein that resolved with 2x SDS-PAGE was transferred onto immunoblot polyvinylidene difluoride membranes (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The blots were blocked with 5% BSA in Tris-buffered saline with 0.1% Tween (TBST) for 2 h at room temperature and were incubated overnight at 4°C with primary antibodies of phospho-p38 MAPK (1 : 1000), phospho-p65 (1 : 1000), phospho-ERK1/2 (1 : 1000), phosphor-IKKα/β (1 : 900), phospho-JNK (1 : 900), phospho-IκB-α (1 : 800), and β-actin (1 : 2000). Blots were then washed three times for 5 min each in TBST and incubated with horseradish peroxidase-labeled secondary goat anti-rabbit antibody at room temperature. After washing for three times, membranes were visualized with Clarity Western ECL Substrate (Bio-rad, Hercules, CA, USA). Densitometry was carried out using Quantity One (version 4.6.2) quantitation software (Bio-Rad, Hercules, CA, USA). All primary antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). β-actin and secondary antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

2.8. Statistical Analysis. Continuous variables were presented as mean ± standard deviation (SD) of 3 independent experiments done in duplicate. One-way analysis of variance (ANOVA) was performed to calculate the significance between groups. Pair-wise comparisons of four data sets were performed using Fisher LSD tests. The Kruskal-Wallis

nonparametric ANOVA test was used for variables that do not follow a normal distribution when comparing multiple groups. A P value of less than 0.05 was considered statistically significant. All statistical analyses were processed using commercially available software package SPSS 20.0 (IBM SPSS Inc., Chicago, IL, USA).

3. Results

3.1. TRQ Protects LPS-Induced Histopathological Damage of Rat Lungs. Histopathological changes in rat lungs showed major difference in gross morphology between groups treated with and without TRQ. In non-LPS-exposed tissues sections, no obvious histological abnormalities were revealed (Figures 1(a)–1(c)). In contrast, intratracheal instillation of LPS induced an acute bronchopneumonia involving the focal areas of the main bronchus and also preterminal bronchioles, presented as prominent thickening of the airway epitheliums and the alveolar septa, conspicuous peribronchial inflammatory cell infiltration, and bronchiolar lumen obstruction by mucus and cell debris. In addition, some blood vessels in affected regions also thickened with a mixed inflammatory infiltrate of main neutrophils and less monocytes and lymphocytes (Figures 1(d)–1(f)). Low dose TRQ treatment failed to contribute to a remarkable alleviation of extensive inflammation with alveolar air spaces flooded with fibrinous exudate admixed with numerous neutrophils at 24 h (Figure 1(g)); however, high dose TRQ expressed noteworthy anti-inflammation property throughout the observing period (Figures 1(j)–1(l)). In general, inflammatory lesion scores decreased over time and they were effectively decreased by TRQ administration in a dose-dependent way (Figure 1(m)).

Besides, a noticeable and conspicuous increase in numbers of mucous cells and amounts of mucosubstances was detected along the airway surface epithelium in LPS group (Figures 2(d)–2(f)), verifying the exact link between airway inflammation and mucus production [31], while the goblet cell metaplasia and hyperplasia were rarely found in the main airways in control rats (Figures 2(a)–2(c)), with a faint positive AB/PAS staining area being detected. TRQ treatment significantly inhibited, though not fully abrogated, the mucus hypersecretion at each time point (Figures 2(g)–2(l)).

3.2. TRQ Regulates LPS-Induced Leukocyte Accumulation in BALF. As a potent stimulus for immune cell, LPS significantly recruited more cells at 24 h, and although the total cell amount decreased over time, it was always significantly higher than that in the control group. TRQ effectively accelerated the process of cell number decrease (Figure 3(a)). For the number of neutrophils, even low dose of TRQ effectively cut down the neutrophil release in BALF after LPS administration at 24 h (Figure 3(b)). The inhibitory effect of TRQ in macrophage accumulation started from 48 h, which was only in high dose group (Figure 3(c)). The number of lymphocytes in BALF enhanced observably after LPS administration, which was completely reversed by high dose TRQ at 24 h (Figure 3(d)).

3.3. TRQ Attenuates LPS-Induced Proinflammatory Cytokines in BALF and Lungs. Since they have been previously reported [32], protein release and mRNA upregulation of TNF-α, IL-1β, IL-6, and CINC-1 were examined to determine the effect of TRQ. As expected, levels of selected cytokines were significantly elevated in BALF (Figure 4) and lung homogenate (Figure 5) 24 h after LPS inoculation. Although they were always significantly higher than those in the control groups, levels of TNF-α, IL-6, and CINC-1 tended to rapidly decrease from 24 h, while levels of IL-1β continued to be high at 48 h. Amounts of TNF-α, IL-6, and CINC-1 were markedly reduced starting from 24 h after LPS exposure by even low dose of TRQ. Of interest, for IL-1β a reduction was observed only in high dose TRQ group at 48 h. At the last time point, high dosage of TRQ successfully recovered levels of all cytokines to normal. Taken together, TRQ exerted a more potent and earlier effect on reversing the overproduction of IL-6, which was completely repressed at 48 h.

As for the qPCR results, CINC-1 was accumulated with mRNA levels peaking at 24 h and then declining. TNF-α, IL-6, and IL-1β mRNA reached a plateau at 24 h and continued to escalate until the end of the 48 hour study. These data demonstrated the complex patterns of cytokine gene expression and suggest that production of early mediators may augment continued expression of TNF-α, IL-6, and IL-1β mRNA. The mRNA induction for each cytokine was significantly mitigated or reversed by TRQ treatment (Figure 6).

3.4. TRQ Modulates LPS-Induced Synthesis of Signaling Proteins in Rat Lungs. Effect of TRQ on LPS-induced p38 MAPK and NF-κB p65 expression in rat lungs was measured by immunohistochemistry. Results revealed that IOD value of areas positively stained by p38 MAPK and NF-κB p65 monoclonal antibody in lung sections increased markedly at 24 h after LPS exposure, which could be significantly attenuated by TRQ in a dose-dependent way (Figures 7 and 8).

3.5. TRQ Modulates LPS-Induced Phosphorylation of Signaling Proteins in MAPKs Pathway in Rat Lungs. Western blot analysis on changes of prominent protein expressions involved in MAPKs pathway in rat lungs showed that stimulation with LPS resulted in a significant increase in the amount of phosphorylation of p38, JNK, and ERK1/2 compared with the control group at 24 h, which were markedly inhibited by an addition of TRQ (Figures 9(a) and 9(c)–9(e)). Except for phospho-JNK, a significant difference between the effects in different dosage of TRQ was observed in all indicators.

3.6. TRQ Suppresses LPS-Induced Phosphorylation of Signaling Proteins in NF-κB Pathway in Rat Lungs. According to the results, stimulation with LPS alone for 24 h notably induced the strong signal of the immunostained band for phosphorylated p65, IκB-α, and IKKα/β in rat lungs, which were significantly decreased after TRQ treatment in a dosage-dependent manner, as expected (Figures 9(b) and 9(f)–9(h)).

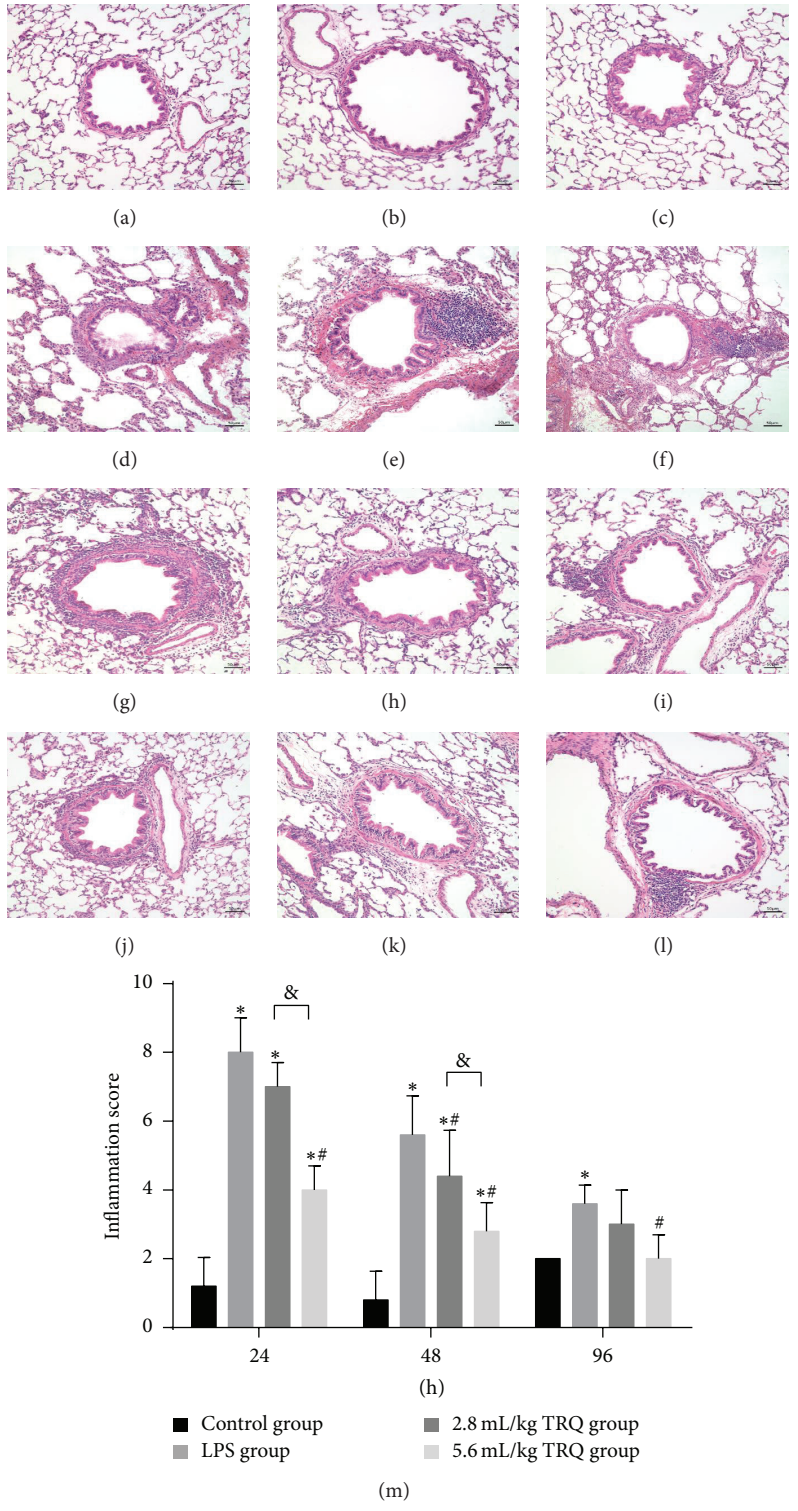

Figure 1: Histological changes in rat airways. Lung tissues from control rats at (a) 24 h, (b) 48 h, and (c) 96 h, rats exposed to LPS alone at (d) 24 h, (e) 48 h, and (f) 96 h, rats treated with LPS + TRQ 2.8 mL/kg at (g) 24 h, (h) 48 h, and (i) 96 h, and rats treated with LPS + TRQ 5.6 mL/kg at (j) 24 h, (k) 48 h, and (l) 96 h were all analysed by haematoxylin and eosin staining. Scale bars = 50 μm. (m) Lung inflammatory scores for rat airways. Values are expressed as mean ± SD. $^{*}P < 0.05$ means significant difference from the control group; $^{\#}P < 0.05$ means significant difference from the LPS group and $^{\&}P < 0.05$ means significant difference between LPS + 2.8 mL/kg TRQ group and LPS + 5.6 mL/kg TRQ group.

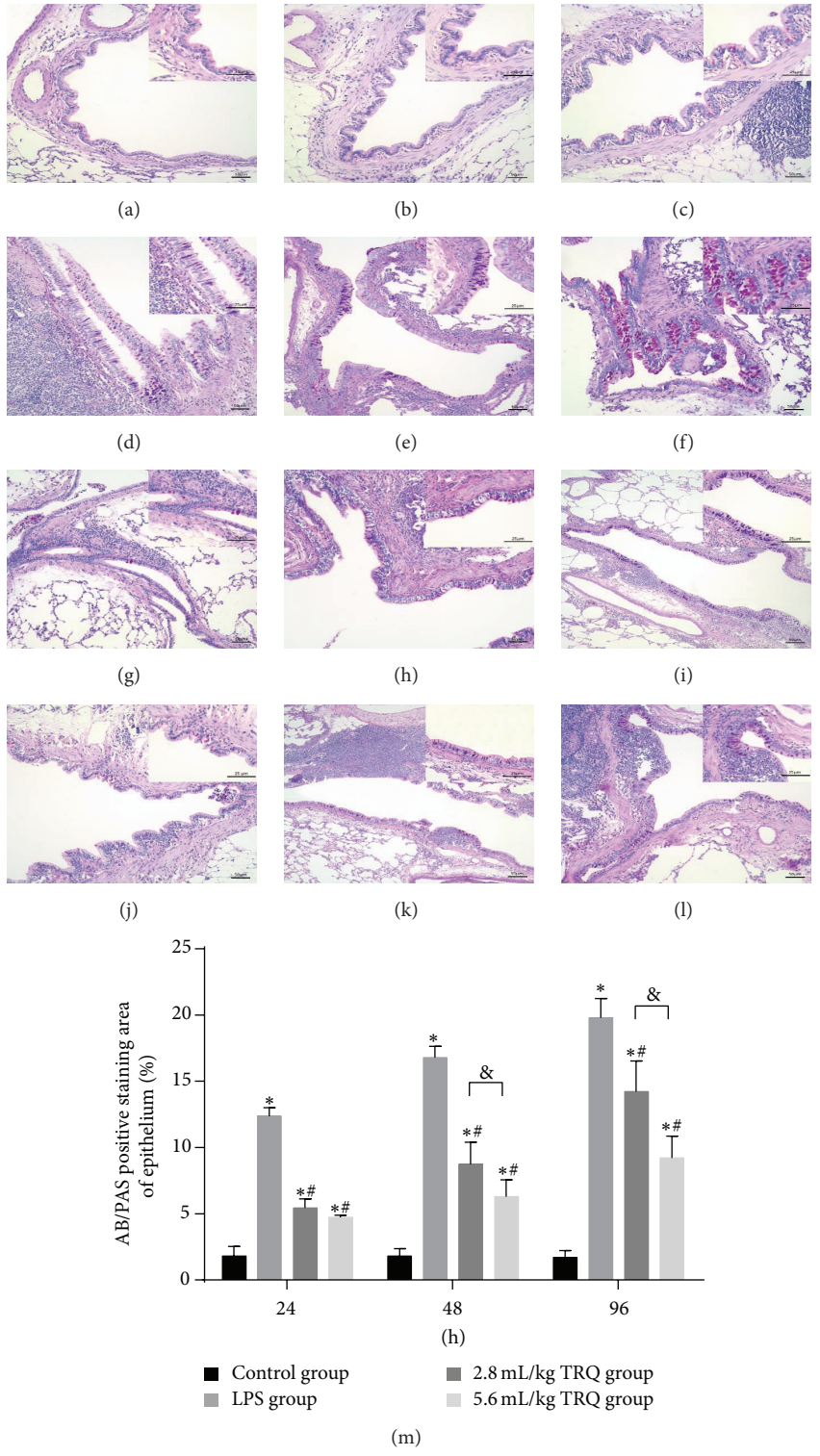

Figure 2: Changes of Alcian blue (AB)/periodic acid-Schiff (PAS) staining in rat airways. Lung tissues from control rats at (a) 24 h, (b) 48 h, and (c) 96 h, rats exposed to LPS alone at (d) 24 h, (e) 48 h, and (f) 96 h, rats treated with LPS + TRQ 2.8 mL/kg at (g) 24 h, (h) 48 h, and (i) 96 h, and rats treated with LPS + TRQ 5.6 mL/kg at (j) 24 h, (k) 48 h, and (l) 96 h were all analysed by AB/PAS staining. Scale bars = 50 μm; upper right insert: scale bars = 25 μm. (m) The percentage of AB/PAS positively staining area to total epithelial area in rat airways. Values are expressed as mean ± SD. $^{*}P < 0.05$ means significant difference from the control group; $^{\#}P < 0.05$ means significant difference from the LPS group and $^{\&}P < 0.05$ means significant difference between LPS + 2.8 mL/kg TRQ group and LPS + 5.6 mL/kg TRQ group.

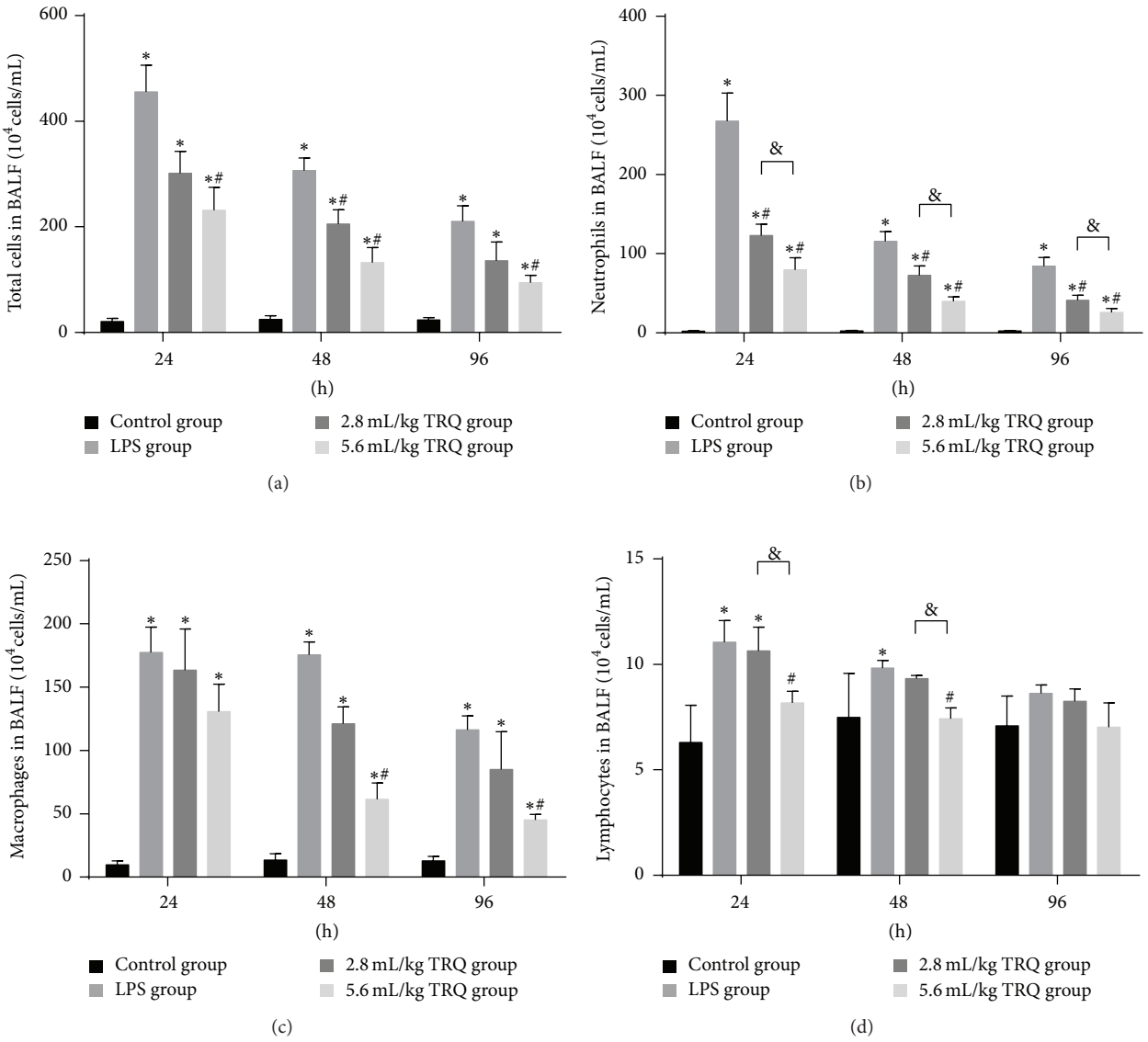

FIGURE 3: Total and differential cell counts in BALF. $^{*}P < 0.05$ means significant difference from the control group; $^{\#}P < 0.05$ means significant difference from the LPS group and $^{\&}P < 0.05$ means significant difference between LPS + 2.8 mL/kg TRQ group and LPS + 5.6 mL/kg TRQ group.

4. Discussion

In recent decades, MAPKs and IKK/IκB-α/NF-κB p65 signaling pathways have attracted considerable attention as targets for inflammation inhibition. In the present study, we focused on the two pathways to tentatively explore the pharmacological mechanisms of TRQ, which, we hope, might in turn advance the drug development. Here we showed that TRQ effectively reduced the phosphorylation of pivotal factors in MAPKs and NF-κB signaling pathways, which contribute to significantly less recruitment and infiltration of immune cells in BALF and subsequent suppression on toxic cytokines release, realizing a promising mitigation on LPS-induced airway inflammation.

As prominent target cells of LPS, neutrophils and macrophages were dramatically induced in BALF after LPS stimulation. As for the neutrophil migration, LPS induces an enhanced chemokinesis by its direct effect on cells and an increased chemotaxis by indirect effects. For example, LPS challenge prominently enhances the level of IL-8, an essential CXC chemokine to attract neutrophils to sites of inflammation in the lung. Both direct and indirect effects are modulated by the coordinated action of ERK, p38 MAPK, and NF-κB pathways [33]. The pathways also contribute to macrophage migration. It has been demonstrated that LPS or TNF-α-induced activation of matrix metalloproteinases, key players in macrophage migration and invasion into foci inflammation, is controlled via ERK1/2, JNK, and

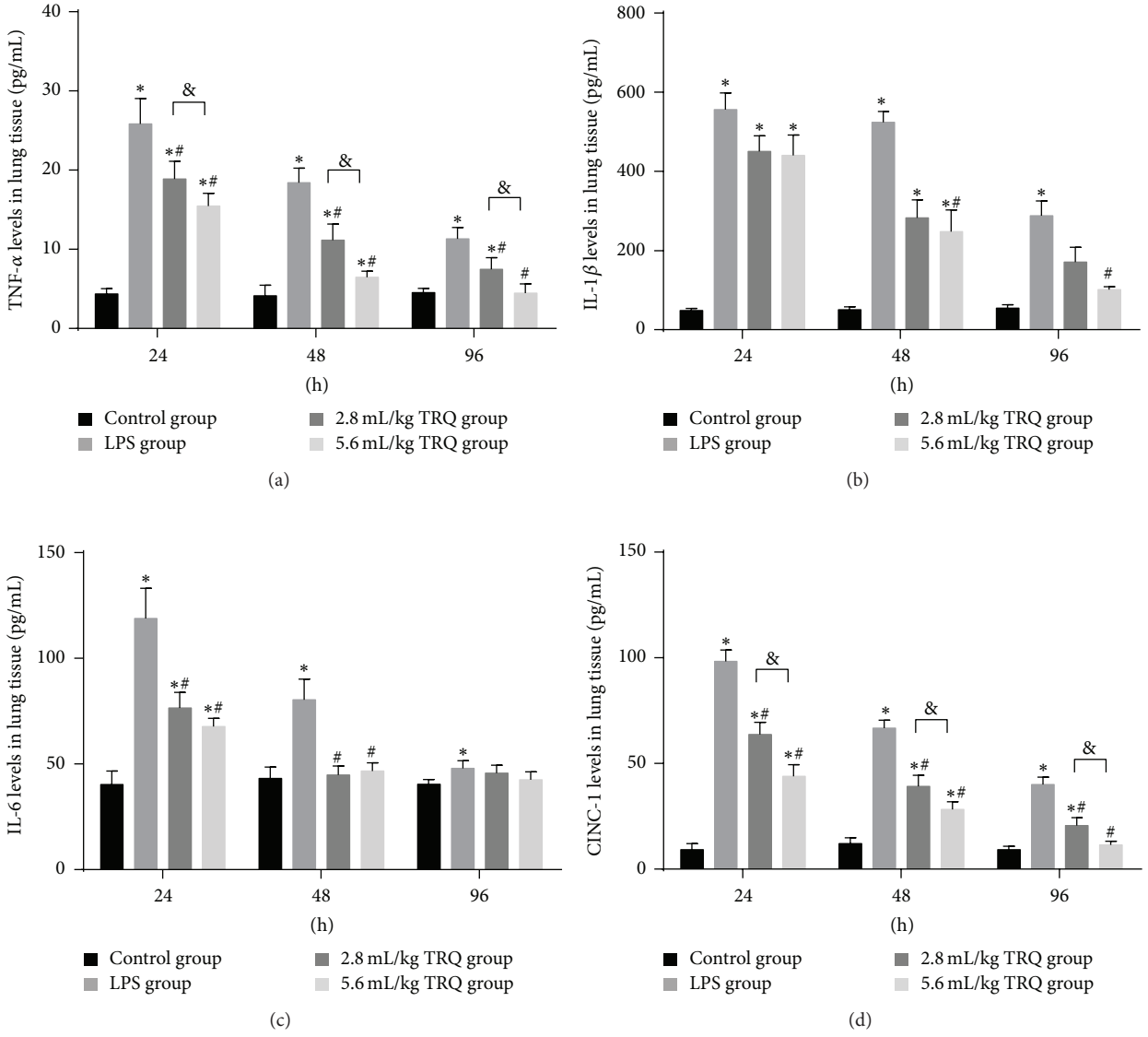

Figure 4: Levels of cytokines in lung tissues. $^{*}P < 0.05$ means significant difference from the control group; $^{\#}P < 0.05$ means significant difference from the LPS group and $^{\&}P < 0.05$ means significant difference between LPS + 2.8 mL/kg TRQ group and LPS + 5.6 mL/kg TRQ group.

IKK/NF-κB pathway [34, 35]. Besides, the expression of MCP-1, another important chemokine for monocytes/macrophages, is stimulated by LPS via NF-κB-dependent mechanism [36]. In particular, the involvement of lymphocytes was also observed after LPS treatment in this study. Previous data showed that lymphocytes present in lung played a role in immunopathology of inflammatory process in both humans and mice [37, 38]. More specifically, study of Dushianthan and colleagues [39] revealed a rapid infiltration of different T cells, including regulatory T cells (Tregs), NKT, and NK cells in human lungs in response to LPS, accompanied by a significant elevation of IL-17, a potent recruiter of neutrophils to inflammatory sites. They demonstrated that both lymphocyte released IL-17 and Tregs modulated the recruitment of neutrophils to the lung in LPS-induced ALI. Therefore, the drastic rise of neutrophils in BALF may partly be attributed to the elevation of lymphocytes in this study.

It has been well characterized that LPS administration on rats leads to overproduction of cytokines from neutrophils and macrophages including IL-1β, IL-6, IL-8, and TNF-α [40–42] for up to 48 h [43], as a result of a series of MAPKs/NF-κB signal transduction cascade. It has been demonstrated that pretreatment with TNF inhibitors led to a reduction in circulating IL-1, IL-6, and IL-8, suggesting an important role of TNF-α in the amplification of inflammatory response [44]. TNF helps neutrophils, monocytes, and lymphocytes recruitment and infiltration by inducing vasodilation and loss of vascular permeability [45] and triggering the secretion of chemokines [46] and cell adhesion molecules [47]. Similarly to TNF, IL-1β is also mainly produced and

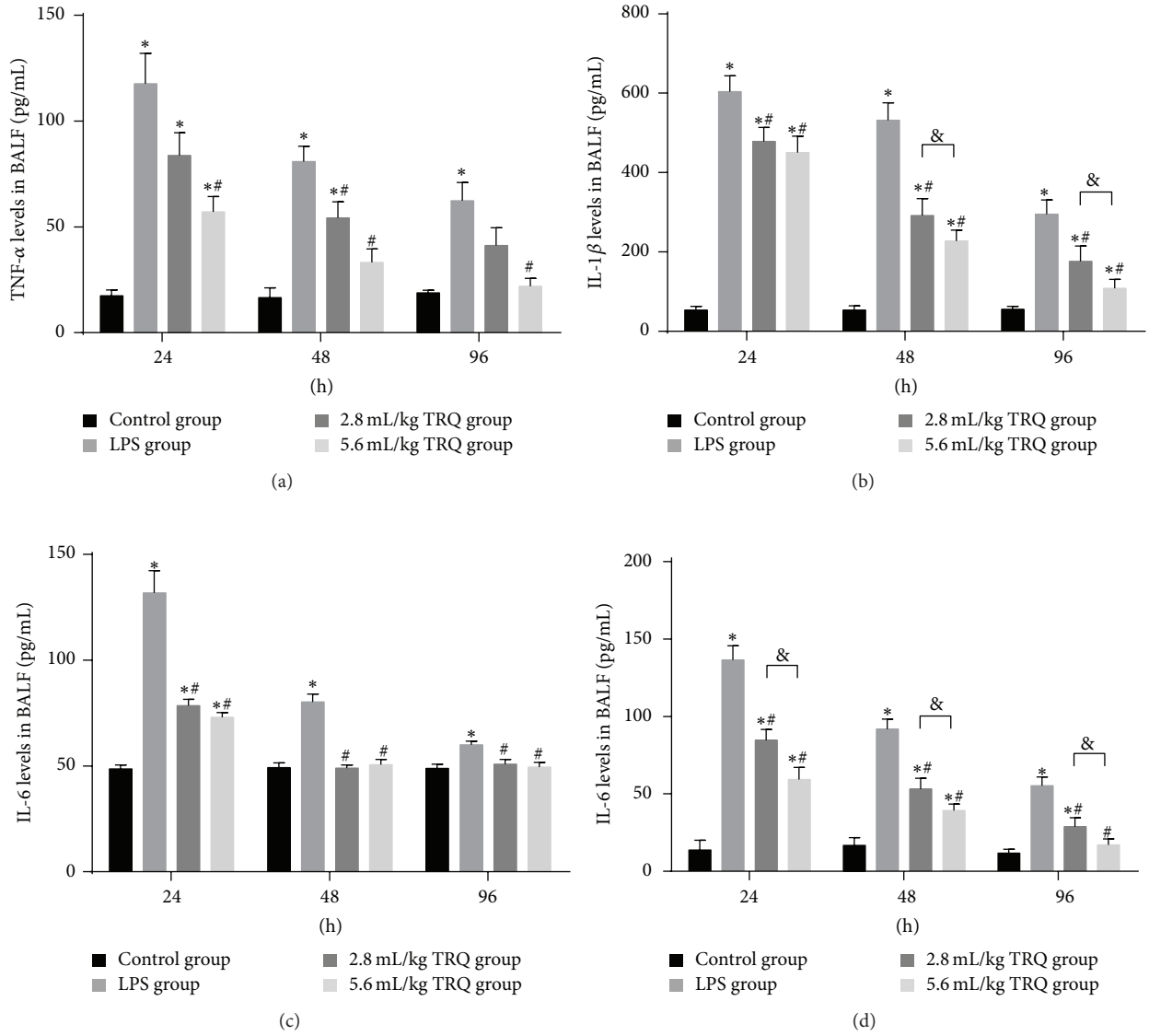

Figure 5: Levels of cytokines in BALF. $^{*}P < 0.05$ means significant difference from the control group; $^{\#}P < 0.05$ means significant difference from the LPS group and $^{\&}P < 0.05$ means significant difference between LPS + 2.8 mL/kg TRQ group and LPS + 5.6 mL/kg TRQ group.

released from monocytes/macrophages in the initiation of inflammatory process after endotoxin exposure. Released IL-1β subsequently triggers further production of IL-1β and other cytokines/chemokines by infiltrating cells and accounts for the perpetuation of inflammation. This may partially explain why IL-1β keeps at high levels at 48 h after LPS instillation in our results. As a pleiotropic cytokine that possesses both pro- and anti-inflammation properties, IL-6 plays a key role in acute-phase of lung injury by affecting the release and functions of neutrophils and macrophages [48]. Time-course studies showed that induction of mRNA for IL-1β and TNF-α occurred rapidly preceding that of IL-6 mRNA after LPS exposure [49]. Subsequently, depending on p38 MAPK and NF-κB pathways, early produced IL-1β and TNF-α induced IL-6 mRNA expression and more IL-6 production. This may explain the continued rise in IL-6 mRNA level till 48 h. Therefore, it is noteworthy that a large proportion of LPS-stimulated IL-6 is actually indirectly induced by TNF-α and/or IL-1β [50]. Inversely, endotoxin-induced IL-6 functions to downregulate its own inducers, TNF-α and IL-1β on mRNA and protein levels [51, 52], with a negative feedback mechanism. As a result, the acute neutrophil and macrophage exudation would be diminished [53], realizing the protective role of IL-6 on endotoxin-induced inflammation [54]. Although nonnegligible amount of IL-6 is induced by TNF-α and IL-1β in this condition, TNF-α and IL-1β are both produced in a very rapid burst in response to LPS; therefore, the decrease of IL-6 levels here was likely not due to a secondary effect exerted by decline of TNF-α and IL-1β levels, which should be ascribed to the effect from

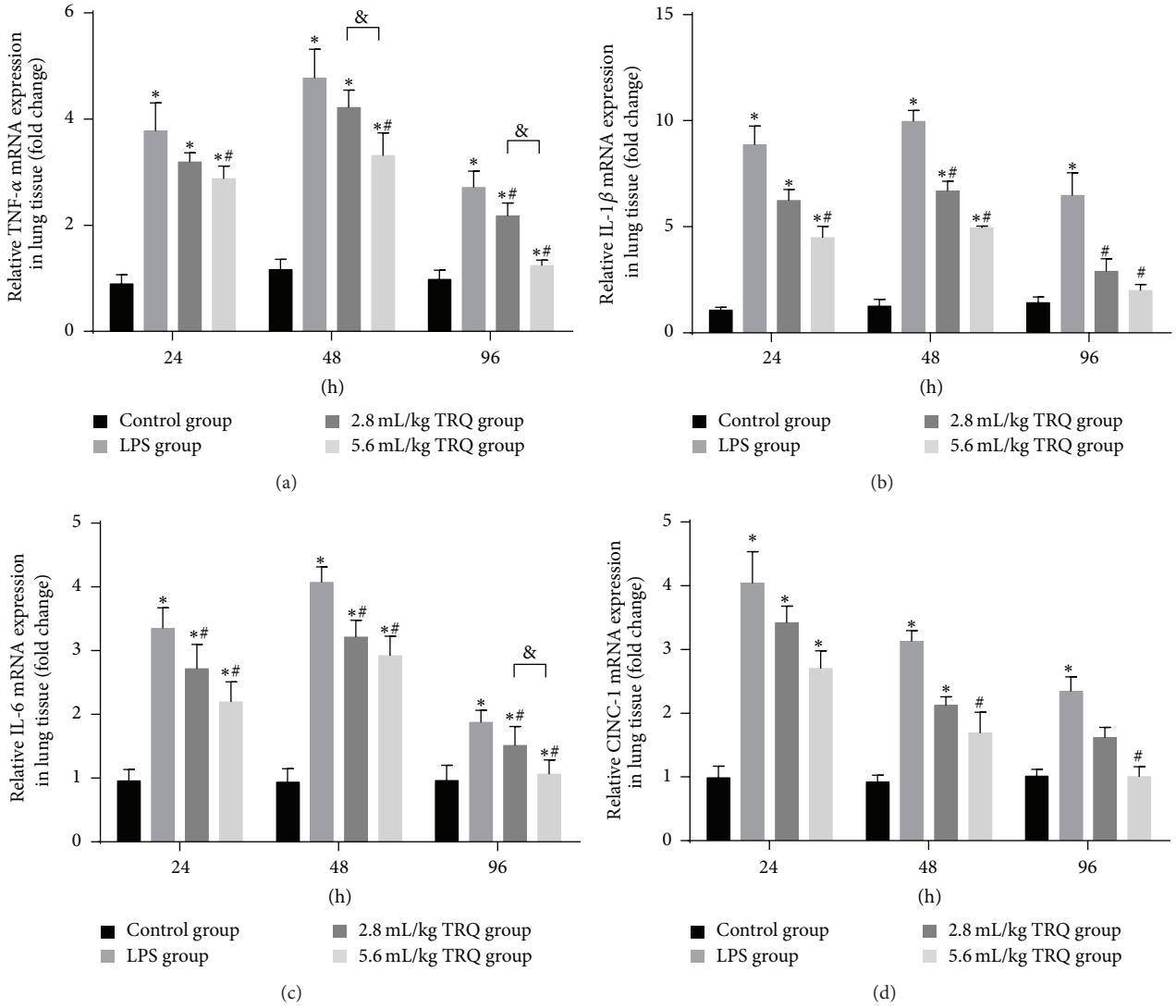

FIGURE 6: Changes in relative mRNA levels of cytokines in lung tissues. $^{*}P < 0.05$ means significant difference from the control group; $^{\#}P < 0.05$ means significant difference from the LPS group and $^{\&}P < 0.05$ means significant difference between LPS + 2.8 mL/kg TRQ group and LPS + 5.6 mL/kg TRQ group.

TRQ. Clinical trials demonstrated that TRQ administration showed a potent effect on lowering the levels of IL-1β, IL-6, IL-8, TNF-α, and IL-17 in plasma of patients under acute pulmonary conditions [11, 55, 56], which was promisingly consistent with our results obtained from rat models.

To our knowledge, it is the first attempt to discover the underlying mechanism involving signaling pathways for the effect of TRQ. This present study had some limitations. Although pathogenesis of LPS-induced airway inflammation generally involves multiple signaling pathways, we only investigated MAPKs/NF-κB pathways and only discussed its role in LPS-stimulated inflammations in vivo. If necessary, we will conduct more comprehensive work in the future to systematically evaluate its function in vitro and multiple pathways and also other pathogen-induced inflammation models.

5. Conclusion

In summary, this work demonstrated that TRQ dose-dependently attenuated LPS-induced neutrophils, macrophages, and lymphocytes infiltration and inhibited proinflammatory cytokines including TNF-α, IL-1β, IL-6, and IL-8 on both mRNA transcription and protein synthesis levels, which were very beneficial to the resolution of inflammation. The therapeutic action of TRQ, in some degree, benefited from its inhibitory effect on the expressions of p38 MAPK and NF-κB p65 in lungs by reducing phosphorylation of p38 MAPK, ERK1/2, IKKα/β, IκB-α, and NF-κB p65. All the present findings specified that the underlying mechanisms of the suppressive actions of TRQ on LPS-induced airway inflammation and mucus overproduction might be due to, at least in part, the suppression on MAPKs and NF-κB signaling pathways.

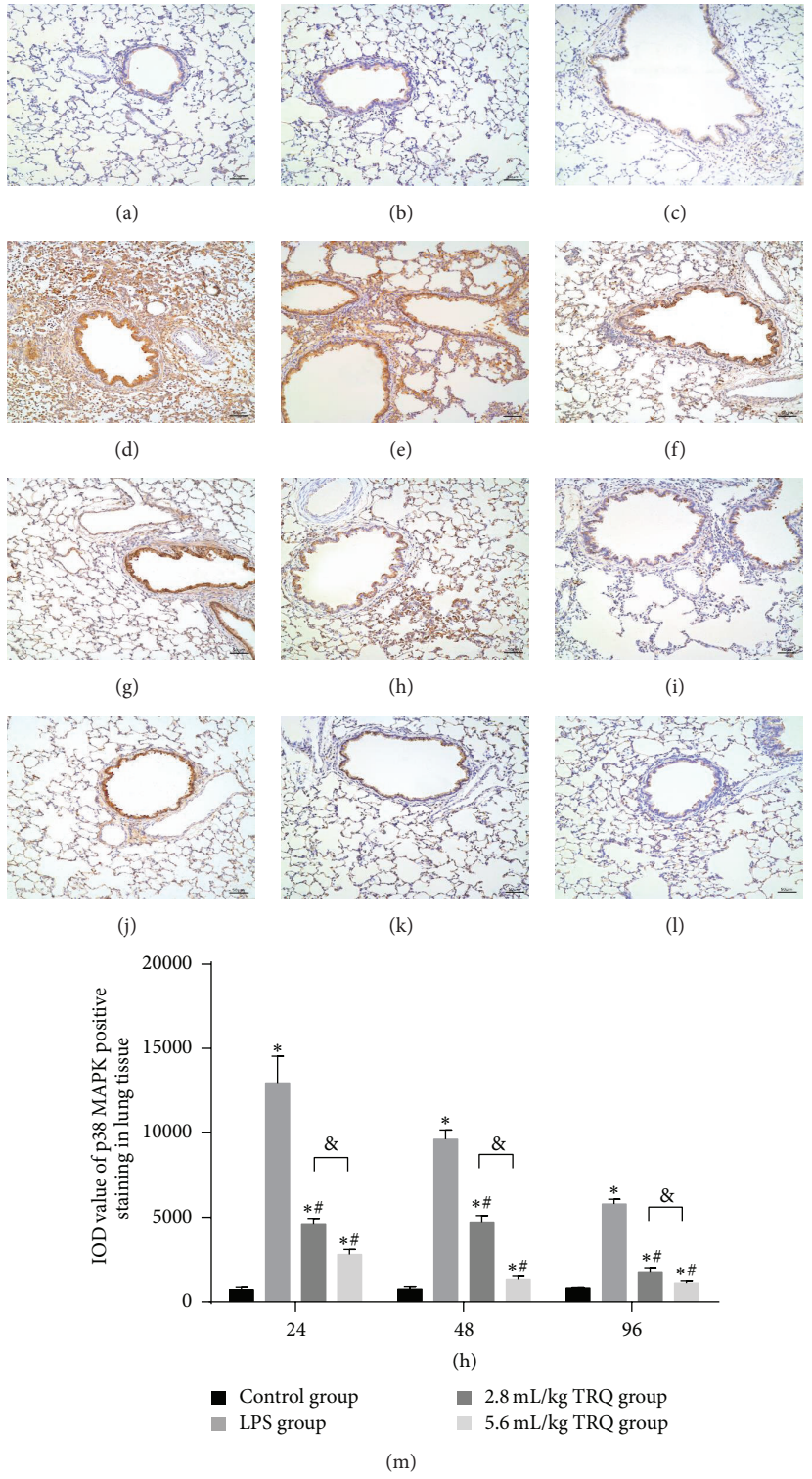

Figure 7: Changes in p38 MAPK immunohistochemical staining in rat airways. Lung tissues from control rats at (a) 24 h, (b) 48 h, and (c) 96 h, rats exposed to LPS alone at (d) 24 h, (e) 48 h, and (f) 96 h, rats treated with LPS + TRQ 2.8 mL/kg at (g) 24 h, (h) 48 h, and (i) 96 h, and rats treated with LPS + TRQ 5.6 mL/kg at (j) 24 h, (k) 48 h, and (l) 96 h were all analysed by haematoxylin and eosin staining. Scale bars 50 μm. (m) Integrated option density sum (IOD sum) value of positive p38 MAPK staining in rats. Values are expressed as mean ± SD. $^{*}P < 0.05$ means significant difference from the control group; $^{\#}P < 0.05$ means significant difference from the LPS group and $^{\&}P < 0.05$ means significant difference between LPS + 2.8 mL/kg TRQ group and LPS + 5.6 mL/kg TRQ group.

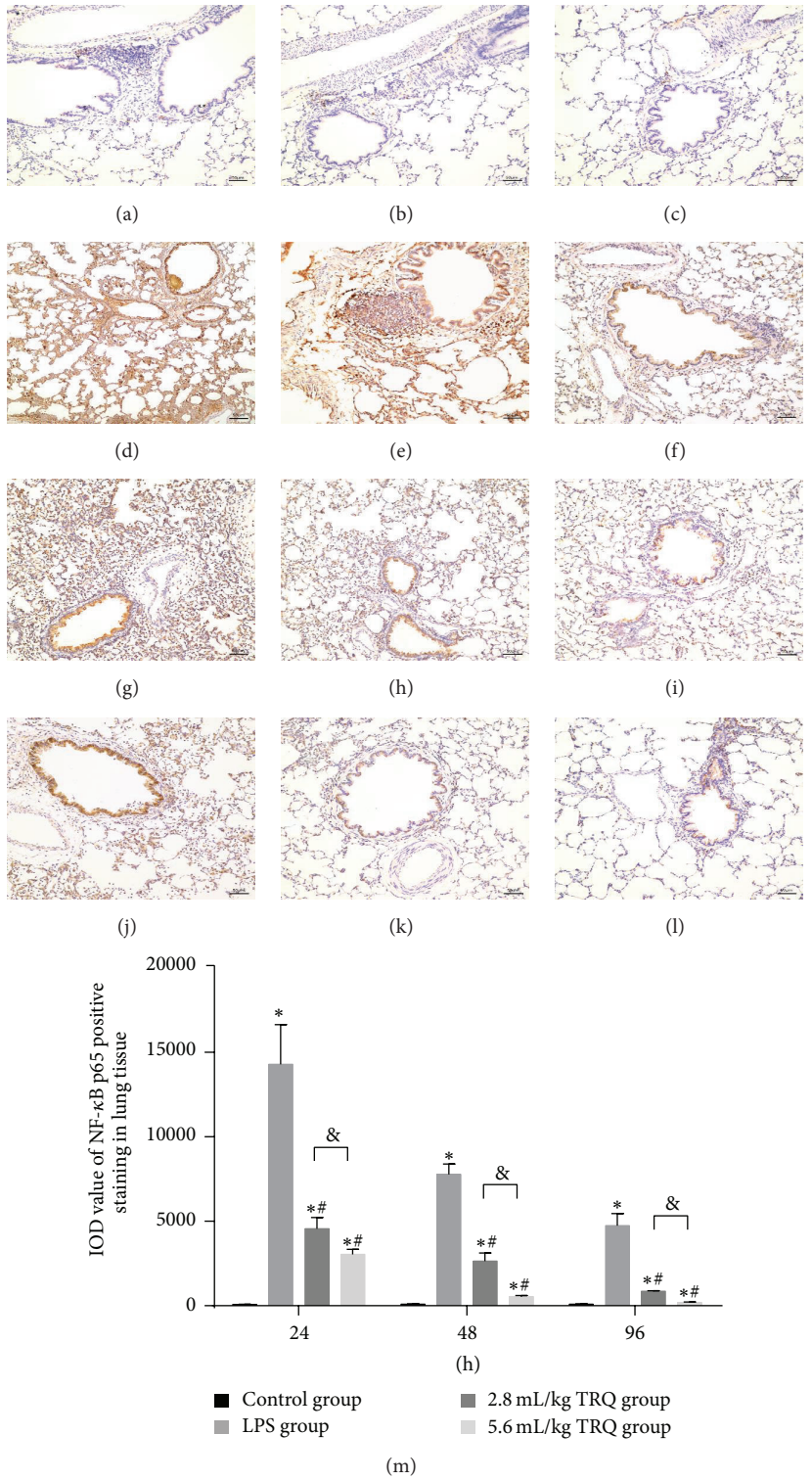

Figure 8: Changes in NF-κB p65 immunohistochemical staining in rat airways. Lung tissues from control rats at (a) 24 h, (b) 48 h, and (c) 96 h, rats exposed to LPS alone at (d) 24 h, (e) 48 h, and (f) 96 h, rats treated with LPS + TRQ 2.8 mL/kg at (g) 24 h, (h) 48 h, and (i) 96 h, and rats treated with LPS + TRQ 5.6 mL/kg at (j) 24 h, (k) 48 h, and (l) 96 h were all analysed by haematoxylin and eosin staining. Scale bars 50 μm. (m) Integrated option density sum (IOD sum) value of positive NF-κB p65 staining in rats. Values are expressed as mean ± SD. $^{*}P < 0.05$ means significant difference from the control group; $^{\#}P < 0.05$ means significant difference from the LPS group and $^{\&}P < 0.05$ means significant difference between LPS + 2.8 mL/kg TRQ group and LPS + 5.6 mL/kg TRQ group.

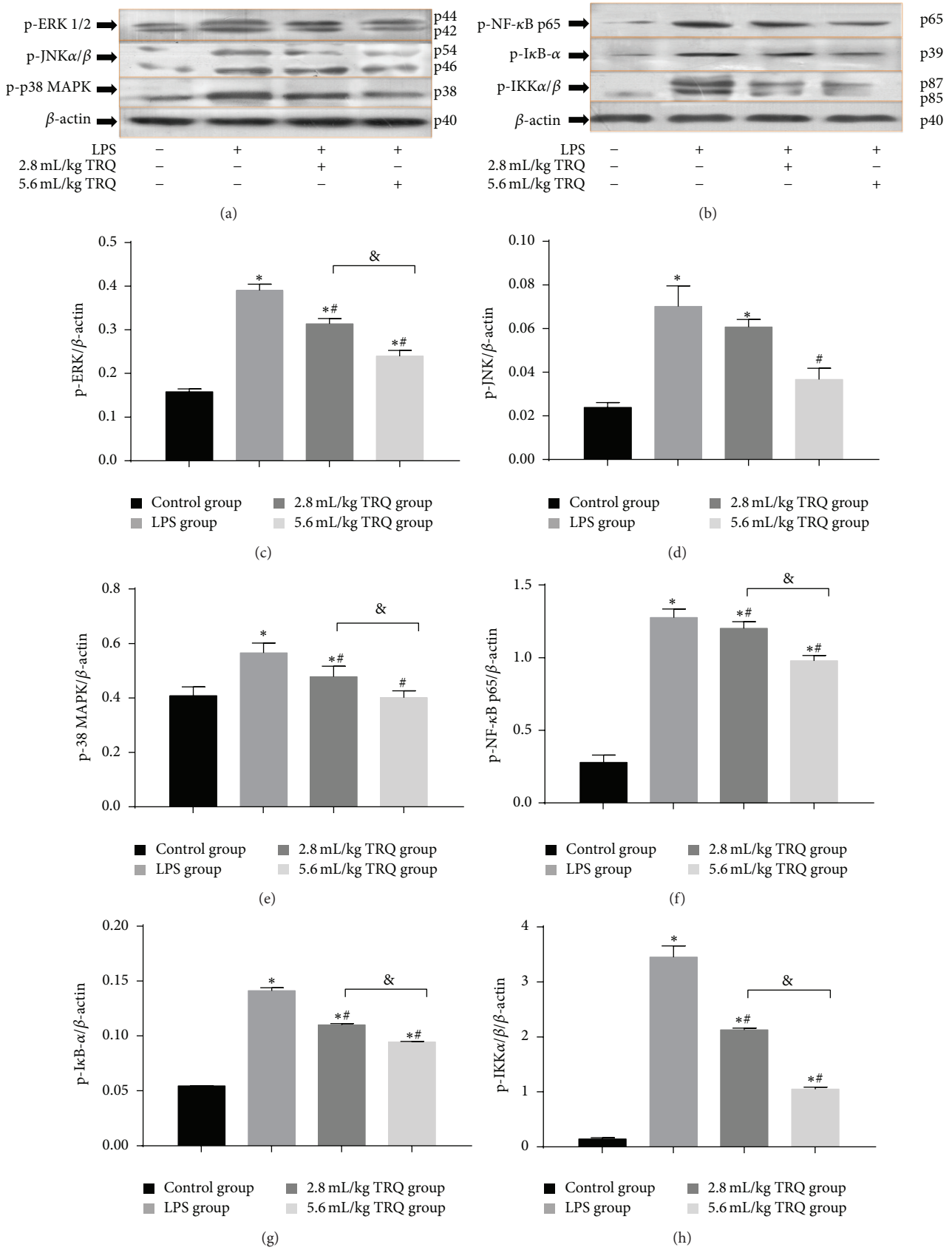

FIGURE 9: Changes in protein levels in lung tissues. $^{*}P < 0.05$ means significant difference from the control group; $^{\#}P < 0.05$ means significant difference from the LPS group and $^{\&}P < 0.05$ means significant difference between LPS + 2.8 mL/kg TRQ group and LPS + 5.6 mL/kg TRQ group.

Competing Interests

The authors declare that they have no competing interests.

Authors' Contributions

Wei Liu and Hong-li Jiang are co-first authors and they contributed equally to this work. Bing Mao contributed to the initiation of the study design. Wei Liu, Lin-li Cai, and Min Yan contributed to lab work implementation. Wei Liu contributed to the draft of the paper. Agreement on the final version was obtained from all authors.

Acknowledgments

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Research Article

Cortex phellodendri Extract Relaxes Airway Smooth Muscle

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Cortex phellodendri is used to reduce fever and remove dampness and toxin. Berberine is an active ingredient of *C. phellodendri*. Berberine from *Argemone ochroleuca* can relax airway smooth muscle (ASM); however, whether the nonberberine component of *C. phellodendri* has similar relaxant action was unclear. An n-butyl alcohol extract of *C. phellodendri* (NBAECP, nonberberine component) was prepared, which completely inhibits high K^+- and acetylcholine- (ACH-) induced precontraction of airway smooth muscle in tracheal rings and lung slices from control and asthmatic mice, respectively. The contraction induced by high K^+ was also blocked by nifedipine, a selective blocker of L-type Ca^{2+} channels. The ACH-induced contraction was partially inhibited by nifedipine and pyrazole 3, an inhibitor of TRPC3 and STIM/Orai channels. Taken together, our data demonstrate that NBAECP can relax ASM by inhibiting L-type Ca^{2+} channels and TRPC3 and/or STIM/Orai channels, suggesting that NBAECP could be developed to a new drug for relieving bronchospasm.

1. Introduction

Asthma is a common chronic respiratory disease [1]. Excessive airway obstruction is a cardinal symptom that results from the contraction of airway smooth muscle (ASM). In this study, we attempted to develop an effective and safe drug from bitter Chinese herbs to inhibit ASM contraction.

Cortex phellodendri, called Huang Bai in Chinese, which tasted bitter, is the dried bark of *Phellodendron chinense* Schneid. or *Phellodendron amurense* Rupr., which belongs to the group of Rutaceae arbor plants. It is bitter in flavor and cold in nature, categorized in kidney, urinary bladder, and large intestine meridians. The traditional functions are to clear heat, dry dampness, purge fire, and remove toxicity. It is one of fundamental traditional Chinese medicines. Previous study reported that *C. phellodendri* has many physiological activities, including antioxidant [2, 3], anti-inflammatory [4–6], antiulcer [7], and immune-stimulating properties [8], as well as neuroprotection and inhibition of coronavirus replication [9, 10]. Moreover, *C. phellodendri* combining with other herbs can reduce complications of corticosteroid-resistant asthma [11]. Berberine is one active ingredient of *C. phellodendri*; berberine from *Argemone ochroleuca* was demonstrated to have a relaxant effect in guinea-pig ASM [12]. However, whether the nonberberine component has similar relaxant action has not been investigated.

In the present study, we found that an n-butyl alcohol extract of *C. phellodendri* (NBAECP, nonberberine component) exerted inhibitory action on ASM contraction, and the underlying mechanism was also investigated.

2. Materials and Methods

2.1. C. phellodendri Extraction. *C. phellodendri*, bark of *Phellodendron chinensis* Schneid. (Rutaceae), were collected in Sichuan Province, China, and were authenticated by Professor Dr. Ding-Rong Wan of our university. A voucher specimen (SCUN201310010) is deposited at the Herbarium of College of Pharmacy, South-Central University for Nationalities, China.

Air-dried *C. phellodendri* (1 Kg) was milled into powder and immersed into 70% ethanol (5 L) for 24 h. The components in the mixture were extracted by hot reflux and were centrifuged. The supernatant was collected and evaporated to dryness under reduced pressure using a rotary evaporator to remove ethanol and get residues, which were immersed in a 2% HCl solution (1000 mL). The yellow precipitates (mainly berberine) were removed and the supernatants were consecutively extracted with petroleum ether, chloroform, ethyl acetate, and n-butyl alcohol. The n-butyl alcohol extract was further evaporated under reduced pressure, and the extraction yield was 1.5% of the raw material dry weight. The dried n-butyl alcohol extract of *C. phellodendri* (NBAECP) was dissolved in 3% DMSO for the experiments.

2.2. Reagents. Nifedipine, acetylcholine chloride (ACH), and pyrazole 3 (Pyr 3) were purchased from Sigma Chemical Co. (St. Louis, MO, USA); DMEM was purchased from Gibco BRL Co. (Invitrogen Life Technologies, Carlsbad, CA, USA). Other chemicals were purchased from Sinopharm Chemical Reagent Co. (Shanghai, China).

2.3. Animals. Sexually mature male BALB/c mice were purchased from the Hubei Provincial Center for Disease Control and Prevention (Wuhan, China). The mice were housed at room temperature (20–25°C) and constant humidity (50–60%) under a 12 h light-dark cycle in an SPF grade animal facility. The experiments on animals were approved by the Animal Care and Ethics Committee of the South-Central University for Nationalities and conformed to the guidelines of the Institutional Animal Care and Use Committee of the South-Central University for Nationalities (QHL-6, 12-10-2013).

2.4. Experimental Asthma Model in Mice. Asthmatic mice were prepared as described previously [13]. Briefly, mice were sensitized by intraperitoneal injection administration of 0.2 mL of 0.9% saline solution containing 0.6 mg OVA and 0.4 mg of adjuvant aluminum hydroxide on days 1 and 8; then, the mice were challenged from days 15 through 19 by daily intranasal instillation of 50 μL of OVA solution (3 mg/mL). Control mice were sensitized and challenged by identical vehicle media.

2.5. Tracheal ASM Contraction Measurement. Mouse ASM contraction was measured as previously described [14]. Adult male BALB/c mice were sacrificed by an intraperitoneal injection of sodium pentobarbital (150 mg/kg), and their tracheae were isolated and quickly transferred to ice cold PSS (composition in mM: NaCl 135, KCl 5, $MgCl_2$ 1, $CaCl_2$ 2, HEPES 10, glucose 10, pH 7.4). The connective tissue was removed, and tracheal rings (~5 mm) were cut from the bottom of the tracheae. Each ring was mounted with a preload of 0.5 g in an organ bath with a 10 mL capacity containing PSS bubbled with 95% O_2 and 5% CO_2 at 37°C. The rings were equilibrated for 60 min, precontracted with high K^+ (80 mM) or ACH (10^{-4} M), washed, and rested for a total of 3 times. The experiments were performed following an additional 30 min rest.

2.6. Bronchial ASM Contraction Measurement. Lung slices were prepared according to a previous report [15]. Lung slices were placed in a chamber and were held with a small nylon mesh. Perfusion was maintained in Hanks' balanced salt solution (HBSS) at a rate of ~800 μL/min. HBSS was supplemented with 20 mM HEPES buffer (composition in mM: NaCl 137.93, KCl 5.33, $NaHCO_3$ 4.17, $CaCl_2$ 1.26, $MgCl_2$ 0.493, $MgSO_4$ 0.407, KH_2PO 0.4414, Na_2HPO4 0.338, and D-glucose 5.56) and adjusted to a pH of 7.4. Images of lung slices under 10x objective were acquired at the rate of 30 frames/min using an LSM 700 laser confocal microscope (Carl Zeiss, Goettingen, Germany). The cross-sectional area of the bronchial lumen was measured using Zen 2010 software (Carl Zeiss, Goettingen, Germany). The experiments were performed at room temperature.

2.7. Data Analysis. The results are expressed as the mean ± SEM. Comparisons of 2 groups were performed using Student's t-test. Differences with $P < 0.05$ were considered significant.

3. Results

3.1. NBAECP Inhibits High K^+-Induced Tracheal Smooth Muscle Contraction. To observe the effect of NBAECP on the contraction of airway smooth muscle (ASM), the tracheal rings (TRs) from healthy mice (i.e., controls) were contracted using high K^+. Following the increase in the K^+ concentration from 10 to 80 mM, the TRs exhibited dose-dependent contraction (Figure 1(a)). Upon the contraction reaching the maximum (at 80 mM K^+), NBAECP was added. The contraction was inhibited in a dose-dependent manner. An identical experiment was also performed in a TR from an asthmatic model mouse, and similar results were observed (Figure 1(b)). NBAECP-induced relaxation in both control and asthmatic TRs was analyzed, and the values of half maximal inhibitory concentration (IC_{50}) were calculated (Figure 1(c)). There were no significant differences between the two traces and the values of IC_{50}. These results indicated that NBAECP could inhibit agonist-induced sustained contraction of control and asthmatic ASM.

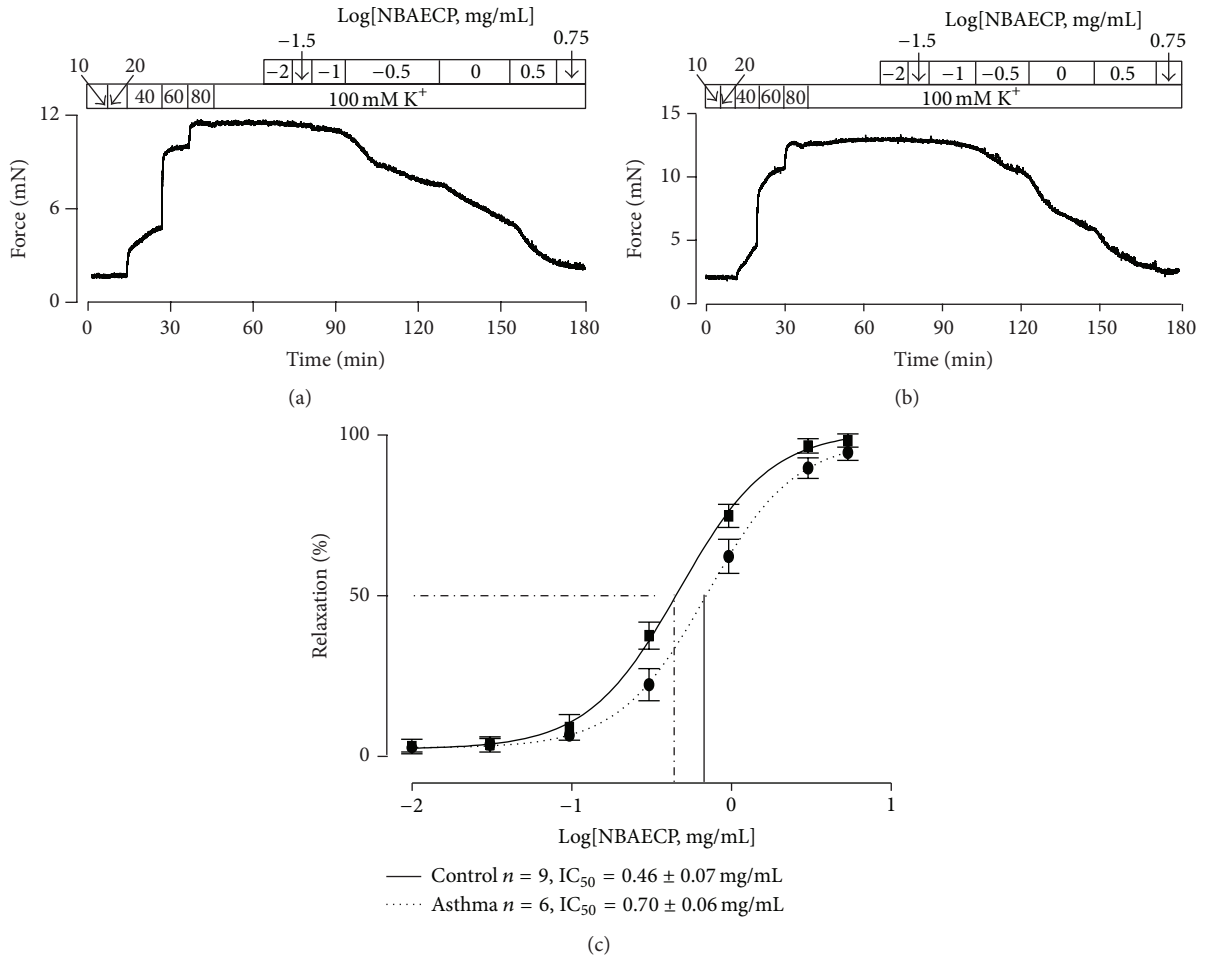

Figure 1: NBAECP inhibits high K^+-induced contraction in TRs. (a) High K^+ triggered contractions in a healthy (i.e., control) TR, which reached the maximum at 80 mM K^+. Following cumulative additions of NBAECP, the sustained contraction was totally blocked. (b) An identical experiment was performed using an asthmatic TR, and a similar result was observed. (c) Dose-relaxation relationships of NBAECP from 9 control and 6 asthmatic TRs. The IC_{50} of NBAECP was 48.9 ± 1.5 μg/mL (n = 9) in control TRs and 73.1 ± 1.6 μg/mL (n = 6) in asthmatic TRs. These data demonstrated that NBAECP could block high K^+-induced precontraction in control and asthmatic tracheal smooth muscle.

3.2. NBAECP Blocks ACH-Induced Tracheal Smooth Muscle Contraction. To know whether NBAECP is capable of inhibiting another agonist-induced precontraction in ASM, healthy TRs were contracted using ACH. Upon the contraction reaching the maximum, NBAECP was added (Figure 2(a)). Similar dose-dependent relaxation responses occurred. Moreover, these responses existed in asthmatic TRs (Figure 2(b)). The dose-relaxation relationships and IC_{50} values of NBAECP were analyzed (Figure 2(c)), and they did not show differences between the control and asthmatic TRs. These experiments demonstrated that NBAECP could also inhibit ACH-induced precontraction in control and asthmatic ASM.

Were these relaxation responses also mediated by L-type Ca^{2+} channels? To answer this question, TRs from healthy and asthmatic mice were precontracted with ACH, and 10 μM nifedipine, a selective blocker of voltage-dependent L-type Ca^{2+} channels (VDCCs), was then added (Figures 3(a) and 3(b)). Following the addition of nifedipine, the contractions were partially inhibited. The resistant components were further blocked by NBAECP. The inhibitions induced by nifedipine and NBAECP were not different between the control and asthmatic TRs (Figure 3(c)).

To further investigate the underlying mechanism of NBAECP-induced relaxation of the nifedipine-resistant component, TRs were incubated with 10 μM nifedipine for 10 min and contracted using ACH, and we then observed the action of Pyr 3 (an inhibitor of TRPC3 and STIM/Orai channels). The results showed that Pyr 3 induced partial relaxation, and the remaining contractions were completely blocked by NBAECP (Figures 4(a) and 4(b)); however, Pyr 3-induced relaxation in asthmatic TRs was markedly greater than in the controls (Figure 4(c)).

Taken together, these results indicated that NBAECP-induced relaxation responses were mediated by L-type Ca^{2+}, TRPC3, and/or STIM/Orai channels.

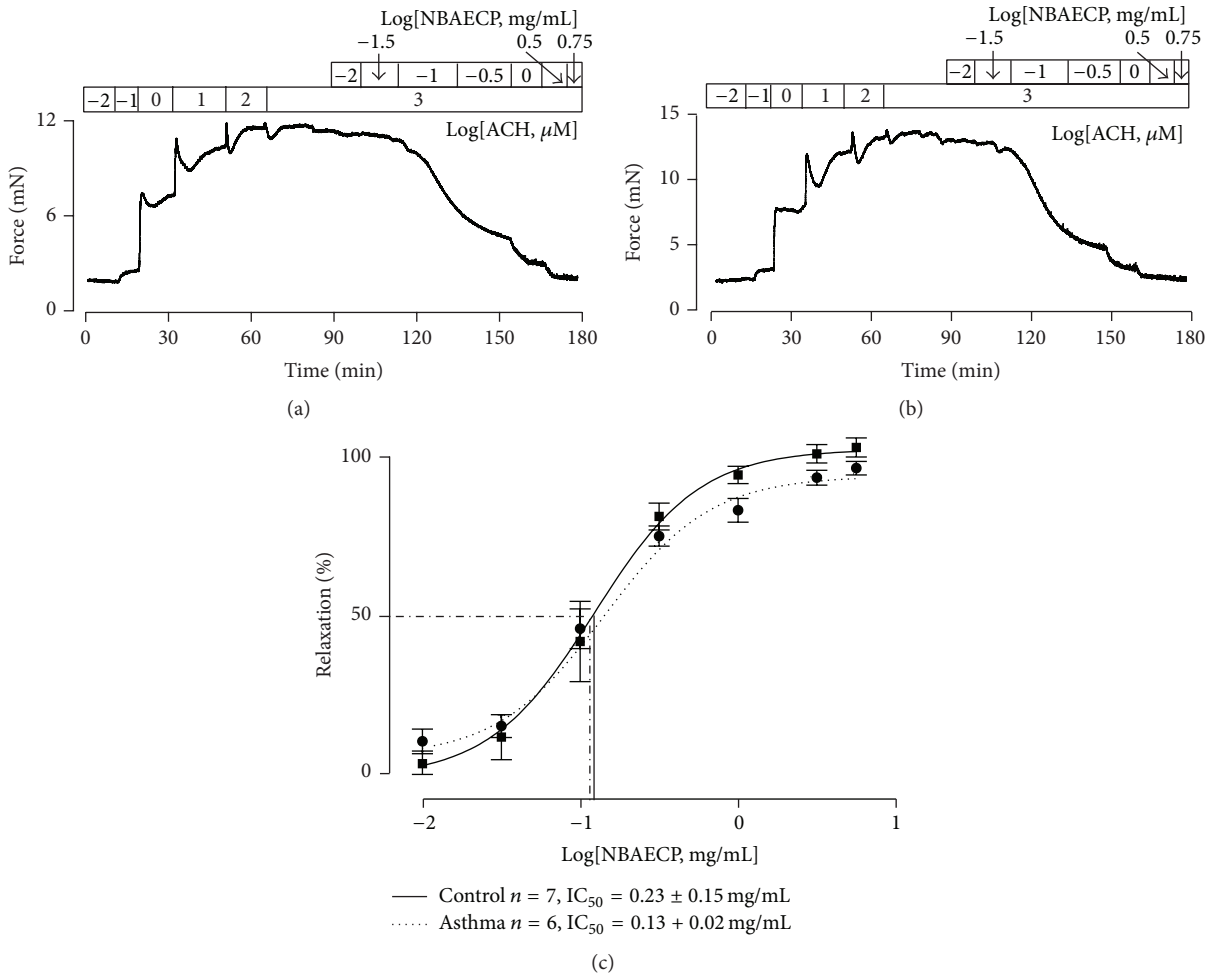

Figure 2: NBAECP inhibits ACH-induced precontraction in TRs. (a) Following cumulative addition of ACH, a TR reached a sustained contraction, which was inhibited following cumulative application of NBAECP. (b) A similar experiment was performed in asthmatic TR. (c) The summary results of NBAECP-induced relaxation in 7 control and 6 asthmatic TRs. The IC_{50} of NBAECP was 12.2 ± 1.3 μg/mL in control TRs and 12.8 ± 1.2 μg/mL in asthmatic TRs. These results indicated that NBAECP could block ACH-induced sustained contractions in control and asthmatic tracheal smooth muscle.

3.3. NBAECP Inhibits Bronchial Smooth Muscle Contraction. To define whether NBAECP has similar relaxant action on bronchial smooth muscle, lung slices were cut, and the cross-sectional area of the airway lumen was measured. The area of the airway lumen from healthy mice markedly decreased following application of 100 μM ACH; however, it was restored upon addition of 3.16 mg/mL NBAECP (Figure 5(a)). Identical experiments were conducted in lung slices from asthmatic mice, and similar phenomena were observed (Figure 5(b)). The summary data are shown in Figure 5(c). These results indicated that NBAECP had similar relaxant action on bronchial smooth muscle.

4. Discussion

In the present study, our data demonstrate that NBAECP can inhibit L-type Ca^{2+} channels, blocking high K^+-induced contractions in healthy and asthmatic airway smooth muscle and additionally inhibiting TRPC3 and/or STIM/Orai channels to reduce ACH-induced contractions in both types of airway smooth muscle. These results indicate that NBAECP could be a new bronchodilator for the treatment of asthma.

The purpose of this study was to find bronchodilators among Chinese medicines. We extracted a component (NBAECP, nonberberine active ingredient) from *C. phellodendri*. To investigate whether NBAECP has relaxant action, we used high K^+ and ACH to contract airway smooth muscle and then observed the effect of NBAECP. NBAECP totally relaxed high K^+-induced precontractions (Figure 1). High K^+ could induce membrane depolarization, resulting in the activation of voltage-dependent L-type Ca^{2+} channels (VDCCs) [16]. The channels then mediated Ca^{2+} influx, resulting in intracellular Ca^{2+} concentration increases, leading to muscle contraction. Nifedipine, a selective blocker of VDCCs, completely blocked high K^+-induced contractions. This phenomenon was observed in our previous study [14, 17]. These results indicated that high K^+-induced contractions completely depended on L-type Ca^{2+} channel-mediated Ca^{2+}

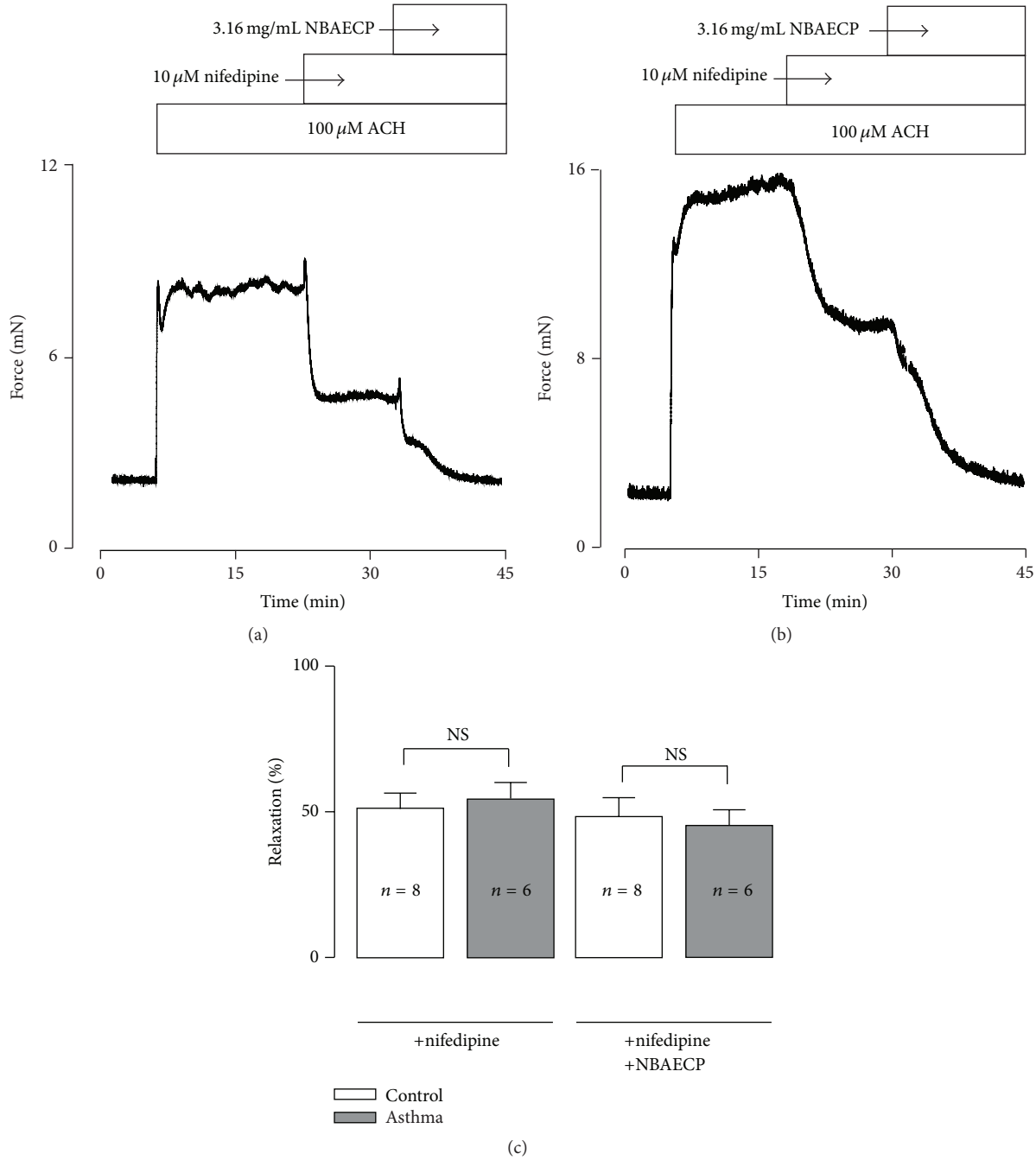

Figure 3: Nifedipine partially inhibits ACH-caused contraction. (a) ACH (100 μM) induced a sustained contraction in a control TR, which was partially inhibited by nifedipine (10 μM). The remaining contract was further blocked by NBAECP (3.16 mg/mL). (b) An identical experiment was performed in an asthmatic TR. (c) The summary data from 8 control and 6 asthmatic TRs. $^{NS}P > 0.05$. These data demonstrated that activation of L-type Ca^{2+} channels played a role in ACH-induced contraction, and NBAECP could inhibit nifedipine-resistant channels, resulting in total relaxation.

influx. Thus, NBAECP-induced complete inhibition of high K^+-induced contraction due to NBAECP resulted in the inhibition of L-type Ca^{2+} channels, thus terminating Ca^{2+} influx. However, the inhibitory mechanism of NBAECP on L-type Ca^{2+} channels must be further investigated.

Airway smooth muscle expresses the muscarinic (M) receptor family, which includes 5 subtypes (M1–M5) [18]. Among them, G protein-coupled M3 plays a more important role in the contraction of airway smooth muscle [19]. Stimulation of M3 by agonists results in intracellular Ca^{2+} release from the sarcoplasmic reticulum (SR) via the PLC-IP3-IP3R pathway [20]. This leads to intracellular Ca^{2+} concentrations increasing and further triggering airway smooth muscle contraction. However, this pathway only mediates

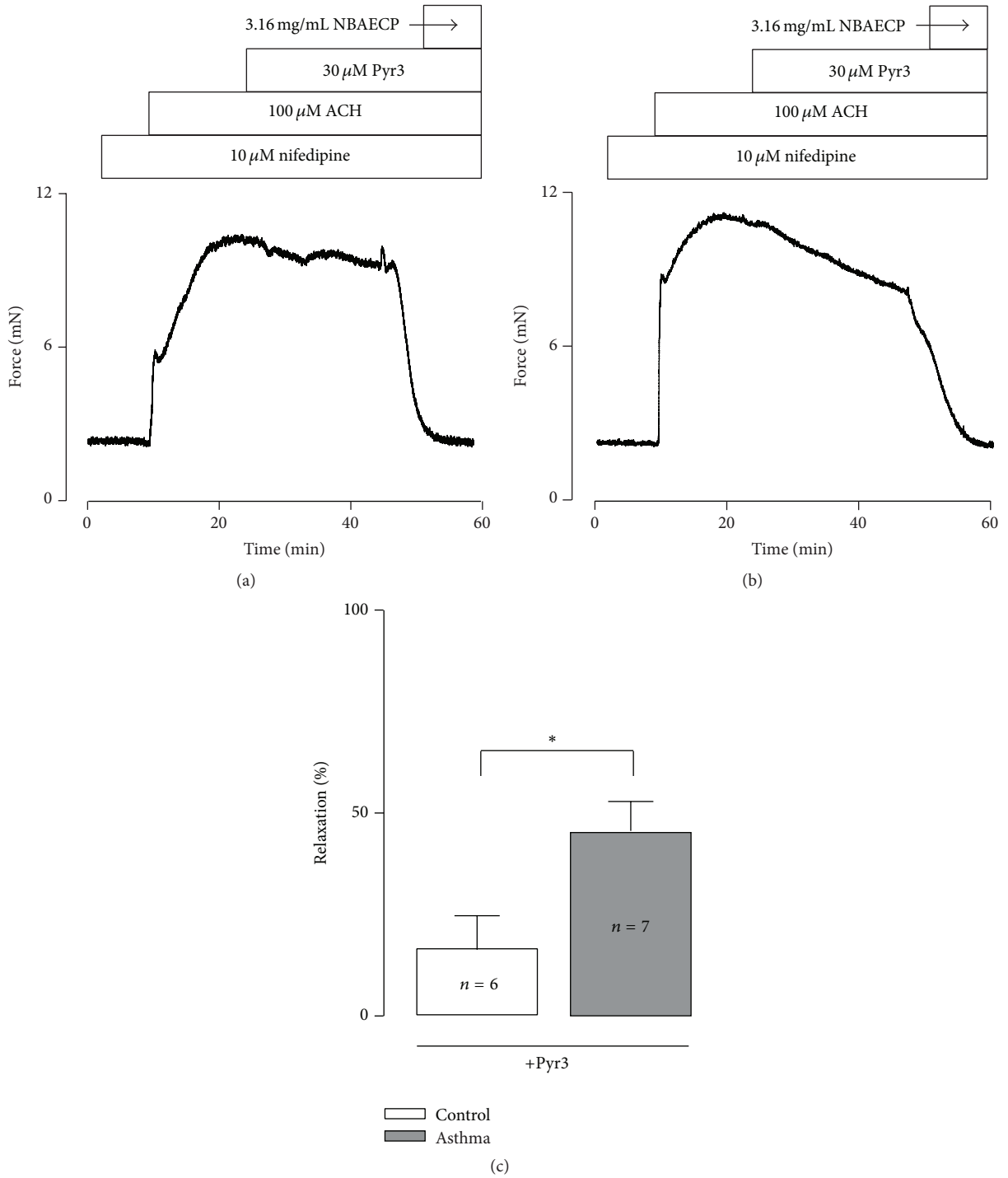

Figure 4: Pyr 3 partially inhibits ACH-induced contraction. (a) In the presence of nifedipine (10 μM), ACH-induced sustained contraction was partially inhibited by Pyr 3 (a blocker of TRPC3 and STIM/Orai channels) and then was completely blocked by NBAECP. (b) The same experiment was performed in an asthmatic TR. (c) Summary results from 6 control and 7 asthmatic TRs. $^*P < 0.05$. These results indicated that activation of TRPC3 and/or STIM/Orai channels participated in ACH-induced contraction, and these channels were inhibited by NBAECP, thus resulting in relaxation.

transient contractions [21], while the sustained contraction depends on Ca^{2+} influx from the extracellular side and/or Ca^{2+} sensitization [22, 23]. Hence, the M3 agonist ACH-induced sustained contraction in airway smooth muscle (Figure 2) was probably due to Ca^{2+} influx. Previous reports have demonstrated that L-type Ca^{2+} channels play roles in ACH-induced airway smooth muscle contraction [24]. These findings were further confirmed in this study, in which

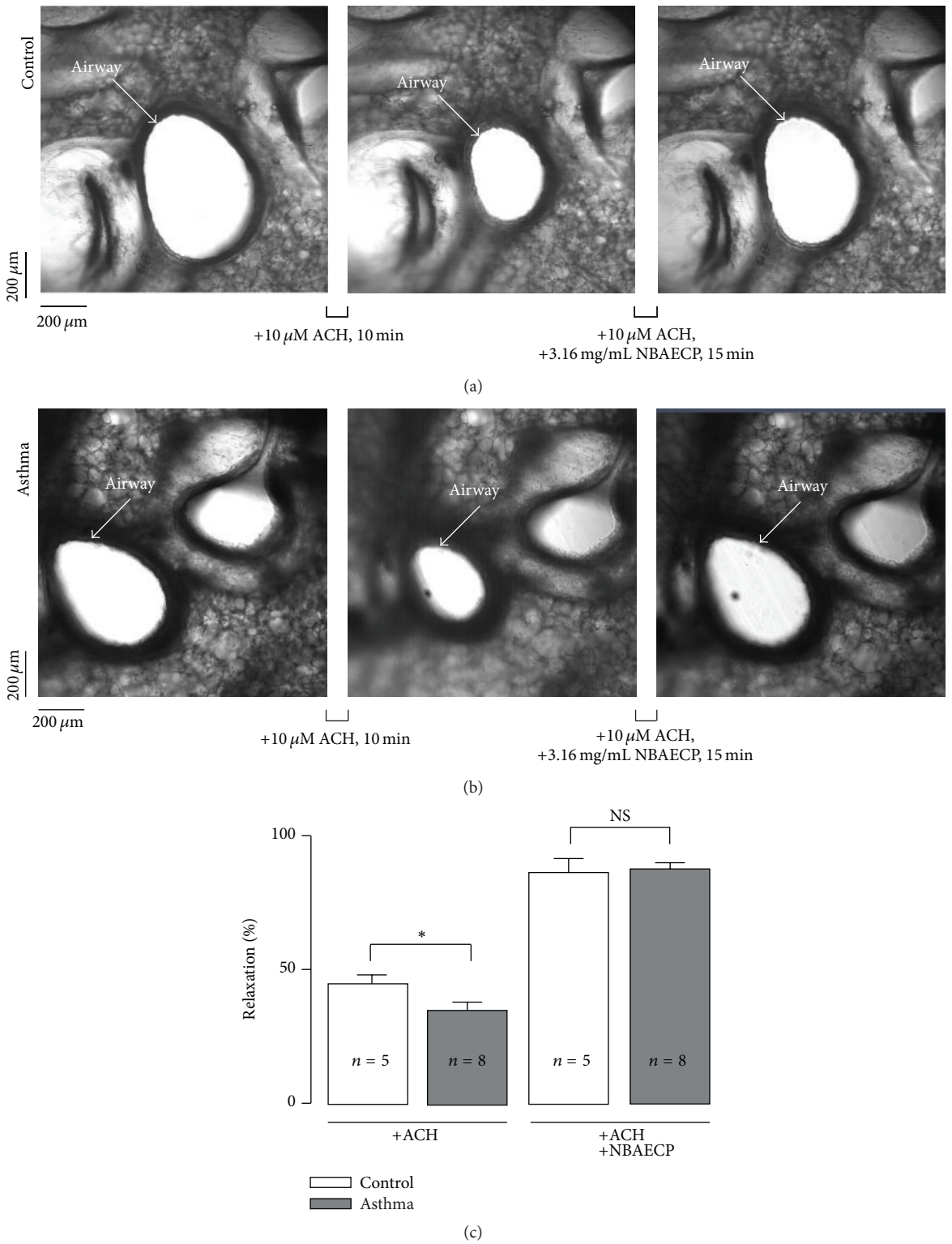

Figure 5: NBAECP inhibits bronchial smooth muscle contraction. (a) The intrapulmonary airway in a lung slice from a control mouse (left) following addition of ACH (10 μM for 10 min): the cross-section area of the airway lumen decreased (middle); then upon application of NBAECP (3.16 mg/mL for 15 min), the cross-section area of the airway lumen increased (right). (b) Similar measurements in an asthmatic lung slice. (c) Summary data from 8 slices/8 control mice and 5 slices/5 asthmatic mice. $^{*}P < 0.05$, $^{NS}P > 0.05$. These experiments demonstrated that NBAECP could inhibit ACH-induced contraction in bronchial smooth muscle.

nifedipine partially inhibited ACH-induced contraction in TRs (Figure 3).

Moreover, TRPC3 and/or STIM/Orai channels also play roles in ACH-induced contractions [14, 25]. TRPC3 and STIM/Orai channels are nonselective cation channels (NSCCs) that can mediate Ca^{2+} influx, resulting in intracellular Ca^{2+} increase to trigger airway smooth muscle contraction and contributing to ACH-induced contractions [26]. In our data, following the addition of Pyr 3 (blocker of TRPC3 and STIM/Orai channels), ACH-induced contractions were partly inhibited (Figure 3). In addition to L-type Ca^{2+} channels and two types of NSCCs, there are still other mechanisms that mediate ACH-induced contraction on the basis of nifedipine-resistant and Pyr 3-resistant contractions being further blocked by NBAECP (Figure 4). These unknown mechanisms must be further determined in the future.

Taken together, the above results indicate that ACH-induced sustained contraction results from L-type Ca^{2+} channels- and TRPC3 and/or STIM/Orai channels-mediated Ca^{2+} influx and unknown mechanisms. Thus, NBAECP-induced inhibition could be partially due to NBAECP inhibiting these channels. However, the detailed inhibitory mechanism requires further investigation.

Although the above data showed that NBAECP could inhibit agonist-induced precontractions in tracheal smooth muscle, whether it has similar inhibitory functions on bronchial smooth muscle is uncertain. Our experiments conducted in lung slices showed that NBAECP was also able to inhibit precontraction in small bronchial smooth muscle (Figure 5), indicating that NBAECP could inhibit whole airway smooth muscle contraction.

In addition, in this study, all of the experiments were performed in both healthy and asthmatic smooth muscles, and similar responses were observed. This finding indicates that NBAECP has similar inhibitory roles in both types of ASM, suggesting that NBAECP could be a potent bronchodilator for asthmatics.

5. Conclusions

NBAECP can inhibit agonist-induced sustained contractions of healthy and asthmatic airway smooth muscle by inhibiting several types of ion channels. These findings indicate that NBAECP could be a new inhibitor of asthma attacks.

Competing Interests

The authors declare no conflict of interests.

Authors' Contributions

Qiu-Ju Jiang, Weiwei Chen, and Hong Dan contributed equally to this work.

Acknowledgments

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Research Article

Effect of Inhalation of Aromatherapy Oil on Patients with Perennial Allergic Rhinitis: A Randomized Controlled Trial

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This study aimed to investigate the effects of aromatherapy oil inhalation on symptoms, quality of life, sleep quality, and fatigue level among adults with perennial allergic rhinitis (PAR). Fifty-four men and women aged between 20 and 60 were randomized to inhale aromatherapy oil containing essential oil from sandalwood, geranium, and *Ravensara* or almond oil (the placebo) for 5 minutes twice daily for 7 days. PAR symptoms determined by Total Nasal Symptom Score (TNSS), the Rhinoconjunctivitis Quality of Life Questionnaire (RQLQ), sleep quality by Verran Synder-Halpern (VSH) scale, and fatigue level by Chalder Fatigue Scale (CFS) were assessed before and after intervention period. Compared with the placebo, the experimental group showed significant improvement in TNSS, especially in nasal obstruction. The aromatherapy group also showed significantly higher improvements in total score of RQLQ and CFS. These findings indicate that inhalation of certain aromatherapy oil helps relieve PAR symptoms, improve rhinitis-specific quality of life, and reduce fatigue in patients with PAR. In conclusion, inhalation of aromatherapy essential oil may have potential as an effective intervention to alleviate PAR.

1. Introduction

Perennial allergic rhinitis (PAR) is a season-independent chronic disorder induced by inflammation mediated by immunoglobulin E (IgE) after allergen exposure, with major symptoms including sneeze, rhinorrhea, and nasal obstruction [1]. It is one of the most frequent chronic diseases, occurring in approximately 500 million people, and causes various impairments including fatigue, cognitive dysfunction, depression, and degraded quality of life [2–5].

The most well-known mechanism of allergic rhinitis (AR) including PAR is antigen-antibody reaction, where allergen-specific sensitization results in mast cell degranulation and the release of inflammatory mediators [6]. Patients with such antigen-specific IgE antibodies present early phase symptoms including sneeze and rhinorrhea and late phase symptoms like nasal obstruction [7]. Though the antigen-antibody mechanism undoubtedly plays an important role in AR, it is not sufficient to explain hypersensitivity to specific chemical mediators and altered organ responsiveness in allergy patients. The recent studies suggested that autonomic nervous system (ANS) dysfunction, especially sympathetic hypofunction, are associated with hypersensitivity of the nasal mucosa in AR [8].

While PAR has diverse medical treatments including avoidance, immunotherapy, pharmacological treatment, and surgery, each of them has its limitations [7]. For example, antigen avoidance is not feasible for those who live in antigen-prone environments, while immunotherapy has adherence issue due to efficacy concerns among patients [1, 9, 10]. Pharmacological treatments including antihistamines and topical steroids may cause sedation and growth problem for children [11].

Despite warnings against their efficacy and side effects, the complementary and alternative medicines (CAM) have gained popularity [12, 13]. Aromatherapy, especially direct inhalation of aroma essential oil fragrance, has long been used for various inflammatory diseases [12]. Essential oils such as eucalyptus, *Ravensara*, and frankincense contain the monoterpenes like 1,8-cineol, alpha-terpineol, and alpha-pinene,

which demonstrate anti-inflammatory and immunomodulatory effect [14–16]. Other essential oils like sandalwood, rich in santalol, are frequently used for relaxation or sedation, implying potential effect on a hypersensitive organ [17].

Despite its advantages and potentials, the aromatherapy has few scientific clinical trials on human PAR patients, especially directly measuring PAR symptoms and quality of life (QOL). The current study aims at exploring the effect of inhaling a mixture of aromatherapy oils on the symptoms and quality of life among PAR patients.

2. Materials and Methods

2.1. Study Design and Participants. A double-blinded, randomized controlled trial was designed to investigate the effects of inhalation of blended aromatherapy oil on subjective symptoms, quality of life, sleep quality, and fatigue in perennial allergic rhinitis patients. The detailed inclusion criteria included (1) age between 20 and 60 years, (2) written diagnosis of PAR by physician, (3) no experience of aromatherapy in the past, (4) no history of psychiatric illness, (5) no current medication or treatment for allergic rhinitis such as surgery and immunotherapy, (6) no disturbance of olfactory acuity, and (7) being free of allergies related to aromatherapy. Ninety men and women aged between 20 and 60 with perennial allergic rhinitis living in Seoul, South Korea, volunteered for the study between June and July 2015; of these, 21 did not meet the eligibility criteria and 7 withdrew their content to participate. The remaining 62 participants were told the purpose and protocol of the experiment and submitted written consent. To ensure all participants are PAR patients, all of the participants presented a written diagnosis from their physicians with a statement that the patients had perennial and chronic AR symptoms and that such diagnosis was based on tests like skin prick test. The study design and protocol were approved by the Ethical Review Committee of the Chung-Ang University (code 1041078-201504-HRBM-083-01), and all participants provided written informed consent.

2.2. Intervention. The subjects were assigned by a random number table into two groups; the experimental group inhaling the fragrance of the blended aromatherapy oil dissolved in almond carrier oil while the control group was inhaling pure almond carrier oil. For the experimental group, aromatherapy oils from three plants, including sandalwood, frankincense, and *Ravensara*, were blended and dissolved in almond oil at a concentration of 0.2% (v/v). For the control group, almond carrier oil was chosen as placebo due to its odorless and nonstimulant attributes [18, 19]. All the aromatherapy oils and almond oil were obtained from Neumond GmbH (Raisting, Germany).

For blinding purpose, only the compounder knew subject assignment, and neither the participants nor the investigators were aware of the allocation. Participants in the two groups received 14 bottles, each containing 1 mL of treatment oil; all bottles had the identical shape and color. Each participant was instructed to self-treat for 14 sessions, performed at 10 AM and 10 PM for 7 consecutive days. They were told to decant the contents of one bottle onto a fragrance pad, sit in a stable and comfortable place, position the pad 30 cm away from their nose, and inhale the fragrance for five minutes with normal breathing [20].

2.3. Outcome Measurements and Data Collection. Pretrial assessments were performed on the day before day 1 of the 7-day intervention, while posttrial assessments were performed on day 8. The pretrial surveys included general characteristics, subjective symptoms by Total Nasal Symptom Score (TNSS), self-assessed qualify of life by Rhinoconjunctivitis Quality of Life Questionnaire (RQLQ), sleep quality measured by Verran Synder-Halpern (VSH) scale, and fatigue by Chalder Fatigue Scale (CFS). Posttrial surveys included all of the above parameters, except for general characteristics. Patients were asked to recall the four previous weeks for baseline measurement during the 7 days of treatment period for posttest measurement. To minimize the effect of data collection time, all surveys and measurements were conducted between 9 AM and 10 AM [20].

2.3.1. PAR Symptoms. As one of the two primary outcome variables for aromatherapy efficacy, mean change in Total Nasal Symptom Score (TNSS) was measured between the baseline and day 8. The current study used the four components of sneeze, rhinorrhea, itchy nose, and nasal obstruction. Each was scored on a 4-point scale from 0 to 3 (0 = none, 1 = mild, 2 = moderate, and 3 = severe) giving TNSS range from 0 to 12 [21].

2.3.2. Disease-Specific Quality of Life (QoL). The other primary outcome measure was the improvement in patients' quality of life, assessed by mean change in Rhinoconjunctivitis Quality of Life Questionnaire (RQLQ) score. RQLQ consists of 28 questions on a 7-point scale (0 = not impaired at all, 6 = severely impaired) in 7 domains: activities limitation, sleep problems, nose symptoms, eye symptoms, non-nose/eye symptoms, practical problems, and emotional function [22]. Total score and seven domain scores were compared.

2.3.3. Sleep Quality. As a secondary outcome measure, this study measured mean change in sleep quality using Verran Synder-Halpern (VSH) scale. VSH consists of eight visual analog scales (VAS) to capture characteristics of sleep latency, fragmentation, length, and depth. Each item is set to a 0–100 response scale. A total score, representative of overall sleep quality, can be calculated by summating each item response [23].

2.3.4. Fatigue. The other secondary outcome measure is mean change in fatigue using Chalder Fatigue Scale (CFS). The CFS is a 14-item questionnaire, each item being scored from 0 to 3, generating a score between 0 and 42 [24].

2.4. Statistical Analysis. All data are presented as mean ± standard deviation, with all statistical analyses were performed using SPSS version 20.0 (SPSS Inc., Chicago, IL, USA). Intergroup comparisons of any normally distributed

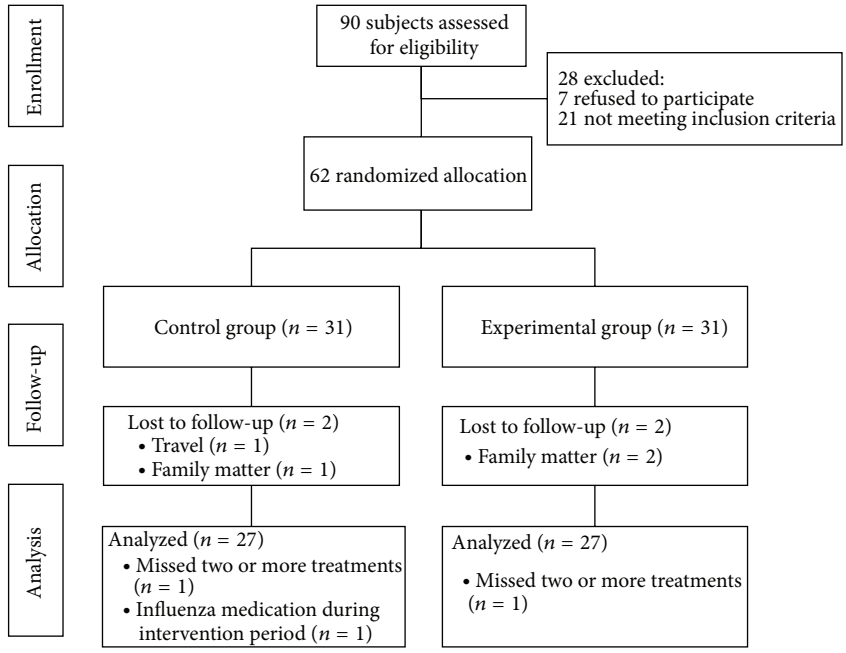

Figure 1: Study flow diagram.

variable were performed using Student's two-sample t-test. Nonnormally distributed variables were compared using the Mann-Whitney U test. Within group, comparisons of normally distributed and non-normally distributed variables were assessed using paired t-tests and Wilcoxon signed rank tests, respectively. A P value < 0.05 was defined as statistically significant.

3. Results

3.1. General Characteristics of the Participants and Test of Homogeneity. From a total of 90 volunteers, 21 did not meet the eligibility criteria and 7 withdrew their content to participate. The remaining 62 patients were randomized and evenly assigned to the control group ($n = 31$) and the experimental group ($n = 31$). Two were lost to follow-up due to travel and family matter from the control group while two were lost to follow-up due to family matter from the experimental group. In addition, four patients were excluded from data analysis due to serious protocol violations: one from the control group and two from the experimental group due to missing two or more treatments and one from the control group due to influenza medication during the intervention [20]. Thus, data were collected and analyzed for 54 men and women, including 27 who received almond oil and 27 who received blended aromatherapy oil (Figure 1). There were no significant differences among the two groups in general characteristics and baseline outcome measures, indicating statistical homogeneity (Table 1).

3.2. Effect of Aromatherapy Oil on PAR Symptoms. After the 7 days of intervention, TNSS in the aromatherapy group reduced significantly more than the almond oil group ($P = 0.022$) while both groups reduced in total score: the aromatherapy group from 6.815 ± 2.202 to 3.259 ± 1.403 and the almond oil group from 6.444 ± 2.532 to 4.593 ± 2.485.

Among the four symptoms, the two groups showed significant difference in the mean change of "nose obstruction" ($P = 0.035$), while "sneeze," "runny nose," and "itchy nose" demonstrated a tendency of higher improvement among the aromatherapy group (Table 2).

3.3. Effect of Aromatherapy Oil on RQLQ. After the intervention, the overall RQLQ score in the experiment group decreased significantly more than the placebo group ($P = 0.002$). Like TNSS, the two groups reduced in the overall RQLQ score: the aromatherapy group from 1.870 ± 0.561 to 0.714 ± 0.436 and the almond oil group from 1.903 ± 0.614 to 1.315 ± 0.770. Out of the seven domains in RQLQ assessment, five domains including practical problem, sleep, nose, activeness, and emotion domains showed significant differences between the two groups. The "eye" and "non-nose/eye physical" domains did not show significant difference though the experimental group had strong tendencies of higher improvement than the control group (Table 3).

3.4. Effect of Aromatherapy Oil on Sleep Quality and Fatigue. The VSH sleep quality score increased in both groups, and there was no significant difference in mean changes between the two groups. In Chalder fatigue score, the experiment group improved significantly more than the placebo group ($P = 0.021$) while both groups reduced in total score: the aromatherapy group from 35.000 ± 7.000 to 23.741 ± 4.703 and the almond oil group from 33.481 ± 7.678 to 27.778 ± 5.938 (Figure 2).

Table 1: Homogeneity test for general characteristics and measurement variables.

Characteristics or variables	Placebo ($N = 27$)	Aromatherapy ($N = 27$)	Total ($N = 54$)	P value
Age (year)	30.852 ± 11.302	28.889 ± 9.010	29.870 ± 10.172	0.652*
TNSS (score)				
Overall	6.444 ± 2.532	6.815 ± 2.202	6.630 ± 2.358	0.569
Sneeze	1.481 ± 0.753	1.519 ± 0.580	1.500 ± 0.666	0.885[a]
Runny nose	1.852 ± 0.864	1.815 ± 0.736	1.833 ± 0.795	0.825[a]
Itchy nose	1.630 ± 0.792	1.630 ± 0.839	1.630 ± 0.808	0.244[a]
Nose obstruction	1.556 ± 0.801	1.852 ± 0.818	1.704 ± 0.816	0.969[a]
RQLQ (score)				
Overall	1.903 ± 0.614	1.870 ± 0.561	1.887 ± 0.583	0.837
Activity limitation	2.605 ± 0.925	2.568 ± 0.772	2.586 ± 0.844	0.924
Sleep problems	1.778 ± 1.013	1.728 ± 1.004	1.753 ± 0.999	0.972[a]
Nose symptoms	2.630 ± 0.861	2.639 ± 0.606	2.634 ± 0.738	0.957
Eye symptoms	1.852 ± 1.057	1.574 ± 0.914	1.713 ± 0.989	0.310
Non nose/eye symptoms	2.185 ± 0.814	1.984 ± 0.743	2.085 ± 0.778	0.390
Practical problems	1.062 ± 0.722	1.358 ± 0.852	1.210 ± 0.796	0.180
Emotional function	0.935 ± 0.903	1.167 ± 0.893	1.051 ± 0.897	0.386
VSH scale (score)	38.570 ± 11.598	42.159 ± 10.025	40.365 ± 10.889	0.229
CFS (score)	33.481 ± 7.678	35.000 ± 7.000	34.241 ± 7.317	0.451

TNSS, total nasal symptoms score; RQLQ, Rhinoconjunctivitis quality of life questionnaire; VSH, Verran Synder-Halpern; CFS, Chalder fatigue scale.
Data reported as mean ± standard deviation.
Student's 2-sample t-test. [a]Mann-Whitney U test.

Table 2: Effect of aromatherapy oil on allergic rhinitis symptoms ($N = 54$).

Characteristics or variables	Placebo ($N = 27$)	Aromatherapy ($N = 27$)	P value
TNSS (score)			
Overall	−1.852 ± 2.125	−3.556 ± 2.486	0.022[a]
Sneeze	−0.370 ± 0.742	−0.630 ± 0.742	0.179[a]
Runny nose	−0.630 ± 0.926	−1.000 ± 0.832	0.219[a]
Itchy nose	−0.556 ± 1.013	−1.074 ± 0.829	0.063[a]
Nose obstruction	−0.370 ± 0.742	−0.852 ± 0.864	0.035[a]

TNSS, total nasal symptoms score.
Data reported as mean ± standard deviation.
Student's 2-sample t-test. [a]Mann-Whitney U test.

Table 3: Effect of aromatherapy oil on allergy rhinitis-specific quality of life ($N = 54$).

Characteristics or variables	Placebo ($N = 27$)	Aromatherapy ($N = 27$)	P value
RQLQ (score)			
Overall	−0.589 ± 0.669	−1.156 ± 0.579	0.002
Activity limitation	−0.589 ± 0.948	−1.281 ± 0.909	0.008
Sleep problems	−0.574 ± 0.865	−1.185 ± 1.000	0.021
Nose symptoms	−0.833 ± 0.805	−1.622 ± 0.850	0.001[a]
Eye symptoms	−0.659 ± 0.925	−1.122 ± 0.771	0.051
Non nose/eye symptoms	−0.722 ± 0.827	−1.119 ± 0.792	0.078
Practical problems	−0.407 ± 0.948	−0.974 ± 0.778	0.020
Emotional function	−0.241 ± 0.944	−0.856 ± 0.943	0.031[a]

RQLQ, Rhinoconjunctivitis quality of life questionnaire.
Data reported as mean ± standard deviation.
Student's 2-sample t-test. [a]Mann-Whitney U test.

4. Discussion

The current study was designed to investigate the effects of aromatherapy oil inhalation on subjective perception of PAR symptoms, quality of life, sleep quality, and fatigue among PAR patients. Fifty-four men and women aged between 20 and 60 years inhaled blended aromatherapy oil or almond oil twice a day for seven days, and the effects on TNSS, RQLQ, VSH, and CFS were measured. Compared with the placebo, the experimental group showed significant improvement in TNSS, RQLQ, and CFS.

TNSS and the "nose" domain of RQLQ directly measure the nasal symptoms of PAR patients. The significant difference in mean change of the two variables indicates that the inhalation of the blended oils helps alleviate PAR symptoms. The three essential oils used for the intervention contain a number of chemical compositions with anti-inflammatory and anti-allergic effect. For example, 1,8-cineole, which is abundant in *Ravensara*, has proven its anti-inflammatory effect by decreasing the production of inflammatory mediators [14, 25, 26]. Alpha-terpineol, another major component of *Ravensara*, also proved its anti-inflammatory effect [27]. Alpha-pinene in Frankincense oil reduces allergic symptoms

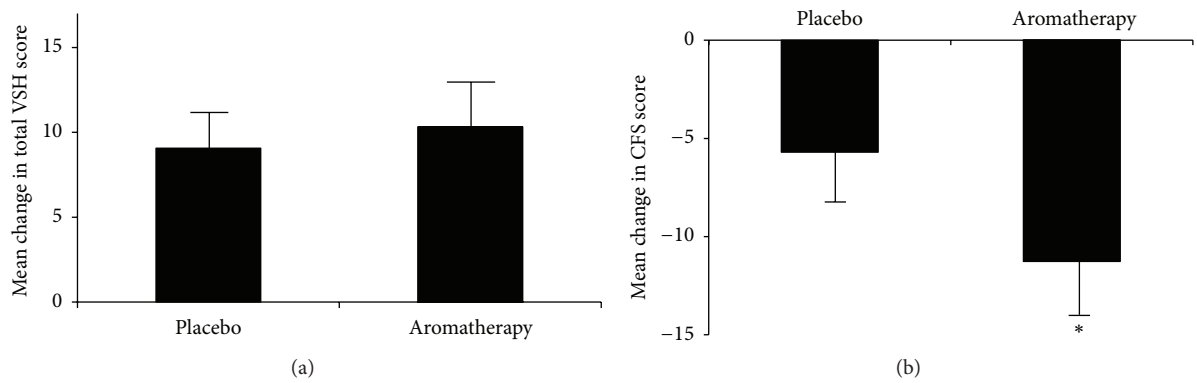

Figure 2: Effect of aromatherapy oil on (a) the Verran Synder-Halpern (VSH) sleep quality scale and (b) Chalder Fatigue Scale (CFS). Values are expressed as mean ± SEM. $^{*}P < 0.05$.

and inflammatory mediator like interleukin-4 (IL-4) among mice with AR [28].

Allergic reaction of AR patients develops in two different patterns along time sequence: the early reaction represented by sneeze and rhinorrhea and the late reaction represented by nasal obstruction [7]. While the early reaction involves stimulated mast cells secreting chemical mediators such as histamines, the late reaction is mainly caused by eosinophil chemotaxis where inflammatory cells migrate to nasal mucosa and remodel normal nasal tissue [7]. From this antigen-antibody reaction perspective, there are two pathways of allergic symptom alleviation: the blended aromatherapy oil reduces the proinflammatory mediators, or it even reduces the number of inflammatory cells.

Looking into the TNSS result, "nasal obstruction" was the only symptom which showed a significant improvement by the aromatherapy oil inhalation. A previous study on patients with allergic rhinopathy showed that a nasal spray containing *Ravensara* essential oil reduced the eosinophils granulocytes and mast cells without eosinophil and metachromatic granules over the intervention period, implying potential for not only soothing AR symptoms but also preventing the source of them [15]. Another study on mice with AR proved that alpha-pinene actually suppressed migration of eosinophils and mast cells [28]. In summary, the blended aromatherapy oil may not only inhibit inflammatory mediator but also recover the sensitized cells.

The effect of the aromatherapy oil on PAR symptoms can be also explained through its effect on autonomic nervous system (ANS) imbalance. Santalol in sandalwood essential oil is associated with elevating parasympathetic nervous system (PNS) for relaxing and sedative effect, and it seems that santalol stimulates parasympathetic nerves in the hypersensitive nasal mucosa for less allergic reactions [29].

Besides the nasal symptoms, the aromatherapy group demonstrated significantly higher improvement in five domains of RQLQ except for "eye" and "non-nose/eye physical" domains. Sandalwood essential oil is known for its "harmonizing" effect, where it relaxes in terms of physiology while it stimulates in terms of behavior [29]. This effect is also demonstrated in the behavioral domains of RQLQ including "activity limitation," "practical problems," and "emotional function."

After the 7-day intervention, the aromatherapy group demonstrated significantly higher improvement in fatigue measured by CFS. In addition, the aromatherapy group improved significantly more than the placebo in the sleep domain of RQLQ and a similar tendency in the VSH score. As PAR symptoms including nasal obstruction cause sleep disorder and fatigue, it can be inferred that alleviated PAR symptoms among the control group help improve sleep quality and reduce their fatigue level [1, 2]. These results are aligned with the previous findings that improvement in AR symptoms would lead to improvement in sleep quality and chronic fatigue is frequently accompanied with AR symptoms [1, 24]. In addition, a number of previous studies have proved that aromatherapy essential oils including lavender, rosemary, and sandalwood help improve sleep quality and decrease fatigue [30].

Comparing the sleep domain in RQLQ survey and the VSH survey, we can find that VSH is inclusive of RQLQ: the questions on time to sleep, sleep fragmentation, and sleep depth are common, while VSH survey adds sleep duration and overall sleep quality. In the current study, the two groups showed significant difference in sleep fragmentation and sleep depth in both questionnaires. In contrast, the two groups were not significantly different in sleep duration, time to sleep, and overall sleep quality. Therefore, the result on sleep quality is generally consistent in both surveys and the apparently conflicting results are mainly due to the difference in survey items.

Reviewing the intragroup comparison across the outcome measures, the control group showed noticeable improvements. This indicates that the placebo effect was not ignorable. Every participant had 14 5-minute inhalation sessions over 7 days, with some lifestyle control like alcohol consumption and smoking. This requires time keeping, stable breathing and inhalation, and self-attentiveness. Stable breathing and inhalation in particular may have relaxation and sedative effect, leading to higher sleep quality [31]. In addition, we can expect some "Hawthorne effects," where the control group shows sizeable improvement without any intervention [32].

In addition, there were some factors which differed between the pretrial environment and the posttrial environment, such as weather, temperature, and humidity. They might affect the intragroup comparison, but not intergroup one.

Most of the previous studies are either animal trials or humans with non-AR diseases, including asthma and bronchitis. Though Remberg's trial involved AR patients treated with 1,8-cineole, it showed only an immediate effect [25]. And most of those studies were conducted in highly controlled environment. In contrast, the current study reports that the inhalation of the aromatherapy oil has a potential for improving AR in human adults over a certain period of time. In addition, these results were obtained in less controlled environment where the participants maintained their normal lifestyle, indicating the practical value of aromatherapy.

The current study has its limitations including (1) no follow-up measurement after posttreatment to investigate the duration of efficacy, (2) no biomarkers for PAR symptoms such as IgE, and (3) aromatherapy efficacy depending on the concentration level. These limitations should be considered in the future studies.

5. Conclusion

In conclusion, the current randomized controlled trial showed that the inhalation of blended oil from *Ravensara*, frankincense, and sandalwood alleviated subjective symptoms, improved the disease-specific quality of life, and reduced fatigue among adult patients with PAR. This intervention also has potential for improvement in sleep quality.

These findings indicate that aromatherapy oil inhalation can be used as a safe and effective complementary intervention to reduce PAR symptoms and improve quality of life among the patients.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

In Vivo Evaluation of the Antiasthmatic, Antitussive, and Expectorant Activities and Chemical Components of Three *Elaeagnus* Leaves

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The leaf of *Elaeagnus lanceolata* and *Elaeagnus henryi* as well as *Elaeagnus pungens* has been documented as an effective herb for the treatment of asthma and chronic bronchitis in traditional clinical medicine. This study was aimed at evaluating the antiasthmatic, antitussive, and expectorant activities of the water extracts from the three plants *in vivo* and analyzing their chemical components by HPLC-DAD. At the medium and high doses, the water extracts of three *Elaeagnus* leaves significantly prolonged the preconvulsive time ($P < 0.01$) in guinea pigs, lengthened the latent period of cough ($P < 0.01$) and decreased the cough frequency caused by aqueous ammonia in mice ($P < 0.01$), and enhanced tracheal phenol red output in mice ($P < 0.01$). There were no significant differences in the pharmacological actions between the three *Elaeagnus* leaves. Moreover, there was more similarity on overlap peaks in the range of retention time from 10 to 40 min by HPLC and many peaks that belonged to flavonoids compounds. It suggested that the main constituents of the three *Elaeagnus* leaves were flavonoid for the pharmacological activities. These effects were the important evidence for the traditional use of *E. henryi* leaf and *E. lanceolata* leaf as well as *E. pungens* to treat asthma and chronic bronchitis.

1. Introduction

Asthma and chronic bronchitis are the chronic inflammatory diseases of the respiratory tract, which are characterized by increased airway hyperresponsiveness and mucus production that leads to episodes of wheezing, coughing, and shortness of breath [1]. Current pharmacological management of these diseases is mainly based on corticoids as anti-inflammatory agents in combination with β_2-adrenergic agonists as bronchodilators [2]. However, these drugs will cause serious side effects. Asthma and chronic bronchitis have a worldwide incidence of 10% in adults and 35% in children. So, the high incidence of the diseases among the individuals presents that research on medications for the repetitious chronic diseases is very important. Particularly, it will be an alternative path to search for effective medicines in the rich traditional medicine in the world [3–5].

The family Elaeagnaceae consists of three genera including *Hippophae*, *Elaeagnus,* and *Shepherdia*. There are about 80 species of *Elaeagnus*, which are widespread in subtropical and temperate areas of East and Southeast Asia. *Elaeagnus pungens* leaf has been documented in the early traditional Chinese medicine (TCM) material medica "Bencao Gangmu" (Ming dynasty, about 430 years ago). It is documented as an antiasthmatic remedy to treat severe asthma, cough, bronchitis, or other respiratory disorders. According to the theory of traditional Chinese herbology in the early material medica "Zhongzang Jing," *Elaeagnus pungens* leaf belongs to the herb of moisturizing lung and cough relieving, and it can astringe the dissipated lung Qi in individuals owing to the acerbity-astringent nature and flavour of the herb.

Through the resource and medical value survey of the *Elaeagnus* plants in minor nationality areas including Hubei province, Tujia nationality, and Guizhou and Yunnan provinces, we found that *Elaeagnus henryi* Warb. ex Diels. and *Elaeagnus lanceolata* Warb. are also generally used as *Elaeagnus pungens* to treat shortness of breath, cough, or bronchitis. On the clinical application, the leaf of the three *Elaeagnus* plants is decocted with water, grinded for powder, or prepared for Chinese patent medicines. Previous researches have

shown that the plants from *Elaeagnus* contain some chemical constituents including flavonoid, lignanoids, organic acids, and terpenoids and have pharmacological effects such as antinociceptive, anti-inflammatory, and cytotoxic actions [6–16]. They have also been verified as nontoxic under oral administration for a long time in adult and in mice [17].

About *E. pungens* leaf, our previous study focuses on the chemical constituents [18, 19], content determination [20], the antiasthmatic, antitussive, and expectorant activities *in vivo* [21], and the relaxant mechanism *in vitro* [22]. Also, we studied the microscopic characteristics of the powders of *E. pungens*, *E. henryi,* and *E. lanceolata* and identified them with infrared spectroscopy [23]. However, whether the leaves of *E. henryi* and *E. lanceolata* have the antiasthmatic, antitussive, and expectorant activities as well as *E. pungens* in animals is still unknown. Furthermore, the relation between the chemical constituents and the active pharmacology effects of the *Elaeagnus* plants has not been investigated. The present study was purposed to compare the activities of the water extracts of three *Elaeagnus* leaves in terms of the antiasthma, antitussive, and expectorant effect *in vivo*. And the chemical components of them were also analyzed by HPLC-DAD. The evaluation will serve as the basis for further research on resources and medical application of the *Elaeagnus* plants.

2. Materials and Methods

2.1. Collection and Preparation of Plant Material. The fresh leaves of *Elaeagnus pungens*, *Elaeagnus lanceolata,* and *Elaeagnus henryi* were collected in October 2014 at Huangmei, Badong, Jianshi, Hubei province, China, respectively. The plants were authenticated by Dr. Dingrong Wan, Professor in Pharmacognosy at School of Pharmacy, South-Central University for Nationalities, with the voucher specimen numbers SCUN 1208002, SCUN 1208004, and SCUN 1208008, respectively. The collected leaves were dried in shade and reduced to coarse powder using a mortar and pestle.

2.2. Extract Preparation. The dried leaves of three *Elaeagnus* plants were extracted with water two times. It was decocted for 1 h each time. The combined solution was filtered and concentrated under reduced pressure to afford the water extract. The yields of the water extract were expressed as the weight percentage of obtained extract in the total weight of crude material, specifically, 22.6%, 17.9%, and 16.9% for *E. pungens*, *E. lanceolata,* and *E. henryi*, respectively.

2.3. Animals and Administration. Guinea pigs of either sex (150–200 g) for antiasthmatic experiments and Kunming mice of either sex (22–25 g) for antitussive and expectorant experiment were purchased from Hubei Province Center for Disease Control and Prevention (Wuhan, China). All animals were housed at room temperature (22–24°C) and constant humidity (50–60%) under a 12 h light-dark cycle in SPF grade laboratory. The animal study was performed according to the international rules considering animal experiments and the internationally accepted ethical principles for laboratory animal use and care.

After 3–5 days of adaptation, the eligible animals were randomly assigned to eleven groups and orally administered, including control group (distilled water), positive group (aminophylline/125 mg/kg, pentoxyverine/50 mg/kg, or ammonium chloride/1000 mg/kg for antiasthmatic, antitussive, or expectorant experiment, resp.), and the water extract groups (low, medium, and high doses). In the tests, administrated dose were 2.7, 5.4, and 10.8 g/kg for guinea pigs and 4.4, 8.8, and 17.6 g/kg for mice (expressed as being equal to the weight of crude material per body weight), which were calculated by coefficient commutation of somatotypes and yield of extract (the used dosage was the medium dose, being five times by clinical dosage of 15 g crude herb in adults). After treatment for 5–7 days, activities were tested and evaluated.

2.4. The Antiasthmatic Test In Vivo. To screen the sensitivity, guinea pigs were placed in a glass chamber (3 L) and sprayed with the mixture of 0.1% histamine and 2% acetylcholine chloride (1 : 1, v/v) under the average pressure of 450 ± 50 mmHg for 15 s [24]. The times to onset of respiratory distress and tumble (preconvulsive time) were recorded. The guinea pigs with preconvulsive time of more than 120 s were considered to be insensitive and discarded. The eligible guinea pigs were randomly allotted to groups ($n = 8$) and administered according to Section 2.3 for 5 days. The administration on day 5 was given at 1.5 h before the measurement of preconvulsive time. The delitescence of convulsion and tumble for each guinea pig within 6 min were observed.

2.5. Antitussive Test In Vivo. To screen the sensitivity, mice were placed in a glass chamber (0.5 L) and sprayed with 25% aqueous ammonia (w/v) under the average pressure of 400 ± 50 mmHg for 5 s. The mice were randomly allotted to eight groups ($n = 10$) and administered according to Section 2.3. All groups were treated with a single dose daily for 7 days and the last dose was given 1.5 h before the measurement of latent period of cough (from the start to the onset of cough) and frequency of cough. The frequency of cough was observed for 2 min.

2.6. Expectorant Test In Vivo. The procedures were performed as described previously [25]. Male and female mice were randomly allotted to eight groups ($n = 10$) and administered according to Section 2.3. All groups were treated with a single dose daily for 5 days and the last dose was given 1 h before intraperitoneal injection of phenol red solution (5% in saline solution, w/v, 0.1 mL/10 g body weight). Then 30 min after application of phenol red, the mice were anesthetized with pentobarbital at the dose of 75 mg/kg body weight and exsanguinated by cutting the abdominal aorta. After being dissected free from adjacent organs, the trachea was removed from the thyroid cartilage to the main stem bronchi and put into 1 mL normal saline immediately. After ultrasonic for 15 min, 1 mL $NaHCO_3$ solution (5%, w/v) was added to the saline and optical density of the mixture was measured at 558 nm using WFZ UV-2000 UV–vis spectrophotometer (Shanghai Spectrum Instrument Co., Ltd., China).

2.7. Chemical Analysis by HPLC/DAD. About 1.0 g of three *Elaeagnus* leaves was immersed in water and decocted for 1 h. The extracted solution was filtered for the tested sample and detected by HPLC-DAD (Model 1200, Agilent Technologies Co. Ltd., USA). An aliquot of the filtrate (20 μL) was injected into a Thermo ODS HYPERSIL C18 column (250 × 4.6 mm i.d., 5 μm) and eluted with a linear gradient with a mobile phase containing solvent A (acetonitrile) and solvent B (0.1% phosphoric acid). The gradient elution program was 5–11% A in 0–5 min, 11–25% A in 5–30 min, 25–36% A in 40–60 min, and 36–80% A in 40–60 min. The flow rate was 1.0 mL/min, the effluent was monitored at 315 nm, and column temperature was set at 30°C. The monomeric compounds of C1 (kaempferol 3-*O*-α-rhamnopyranosyl-(1 → 2)[α-rhamnopyranosyl-(1 → 6)]-β-D-galactopyranoside), C2 (kaempferol 3-*O*-α-L-rhamnopyranosyl-(1 → 2)-β-D-glucopyranoside), and C3 (kaempferol 3-*O*-(6″-*O*-*E*-*p*-coumaryl)-β-D-glucopyranoside) were also injected as the standard substances.

2.8. Statistical Analysis. Data obtained in all experiments was expressed as mean ± SD. Statistical analysis was done by one-way analysis of variance (ANOVA) with Tukey test using the software of Origin 7.0. Differences between means of treated groups and the control were regarded as significant at $P < 0.05$.

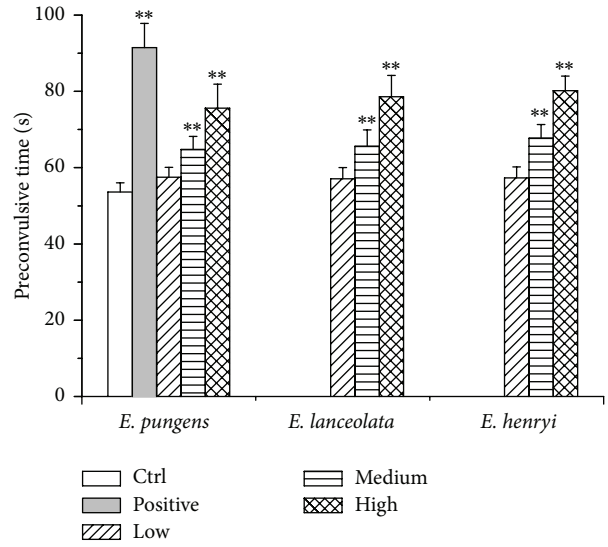

Figure 1: Effect of control, positive (aminophylline 125 mg/kg), water extracts of *E. pungens*, *E. lanceolata,* and *E. henryi* leaf (low, medium, and high doses of 2.7, 5.4, and 10.8 g/kg, expressed as being equal to the weight of crude material per body weight) on guinea pigs bronchoconstriction induced by mixture spraying histamine and acetylcholine chloride after administration for 5 days ($n = 8$). Values are presented as mean ± SD, $^{**}P < 0.01$, compared with control group.

3. Results

3.1. Antiasthmatic Effects. The effects of the water extracts from three *Elaeagnus* leaves in guinea pigs exposed to mixture spray of 0.1% histamine and 2% acetylcholine chloride were shown in Figure 1. The preconvulsive times of eleven groups had no difference before administration ($P > 0.05$). After administration, the preconvulsive times were 53.6 ± 2.4, 91.5 ± 6.3, 57.5 ± 2.6, 64.8 ± 3.4, 75.6 ± 6.3, 57.1 ± 2.9, 65.6 ± 4.3, 78.6 ± 5.6, 57.3 ± 2.9, 67.7 ± 3.6, and 80.2 ± 3.8 s in the control, aminophylline, and low, medium, and high dose of the water extracts from the leaves of *E. pungens*, *E. lanceolata,* and *E. henryi* groups, respectively. It showed that aminophylline and medium and high dose of water extracts from the three *Elaeagnus* leaves increased the preconvulsive time by 70.71%, 20.90%, 41.05%, 22.39%, 46.64%, 26.31%, and 49.63%, respectively. Comparing with control group, there were very significant differences in aminophylline group and medium and high dose groups of tested extracts ($P < 0.01$) by ANOVA among all the groups.

3.2. Antitussive Effects. The antitussive effects of the water extracts from three *Elaeagnus* leaves on mice were shown in Figures 2(a) and 2(b). All water extracts induced latent period of cough and reduced cough frequency in a dose-dependent manner. Comparing with control group, there were significant differences in positive and medium and high dose groups of tested extracts ($P < 0.01$). The percentage of latent period of cough increased 121.72% (pentoxyverine), 35.21%, 112.36% (*E. pungens*), 86.52%, 119.10% (*E. lanceolata*), and 63.67%, 111.99% (*E. henryi*), respectively. And cough frequency was inhibited by 45.45% (pentoxyverine), 19.96%, 38.68% (*E. pungens*), 24.24%, 41.18% (*E. henryi*), and 26.2%, 42.42% (*E. henryi*), respectively.

3.3. Expectorant Effects. As shown in Figure 3, both positive and other tested groups prompted tracheal phenol red output after administration. It presented that the tracheal phenol red output in ammonium chloride and medium and high dose of water extracts from the leaves of *E. pungens*, *E. lanceolata,* and *E. henryi* groups increased by 6.87, 0.56, 1.18, 3.92, 0.87, 1.79, 3.59, 0.56, 1.82, and 3.79 folders, respectively. Except the low doses of three extracts and medium dose of *E. pungens*, the differences showed significantly in medium doses of other two plants ($P < 0.05$) and high doses of three plants ($P < 0.01$).

3.4. Chemical Analysis. The chromatographies of *E. pungens*, *E. lanceolata,* and *E. henryi* leaves by HPLC were shown in Figures 4–6. We found that the peaks at the retention time (t_R) of 20.650 min and 38.733 min in the *E. pungens* leaf (Figure 4) were consistent with the monomeric compounds of C1 (kaempferol 3-*O*-α-rhamnopyranosyl-(1 → 2)[α-rhamnopyranosyl-(1 → 6)]-β-D-galactopyranoside, t_R = 20.600 min) and C3 (kaempferol 3-*O*-(6″-*O*-*E*-*p*-coumaryl)-β-D-glucopyranoside, t_R = 38.763 min), respectively. Compared to the monomeric compounds, the overlap ratio of the absorptive curves by DAD was up to 0.97~0.99. In Figure 5, the overlap peak was t_R 38.797 min (C3) in the *E. lanceolata* leaf and three overlaps peaks was t_R 20.683 min (C1), 23.157 min (C2, kaempferol 3-*O*-α-L-rhamnopyranosyl-(1 → 2)-β-D-glucopyranoside, t_R = 23.100 min), and 38.87 min

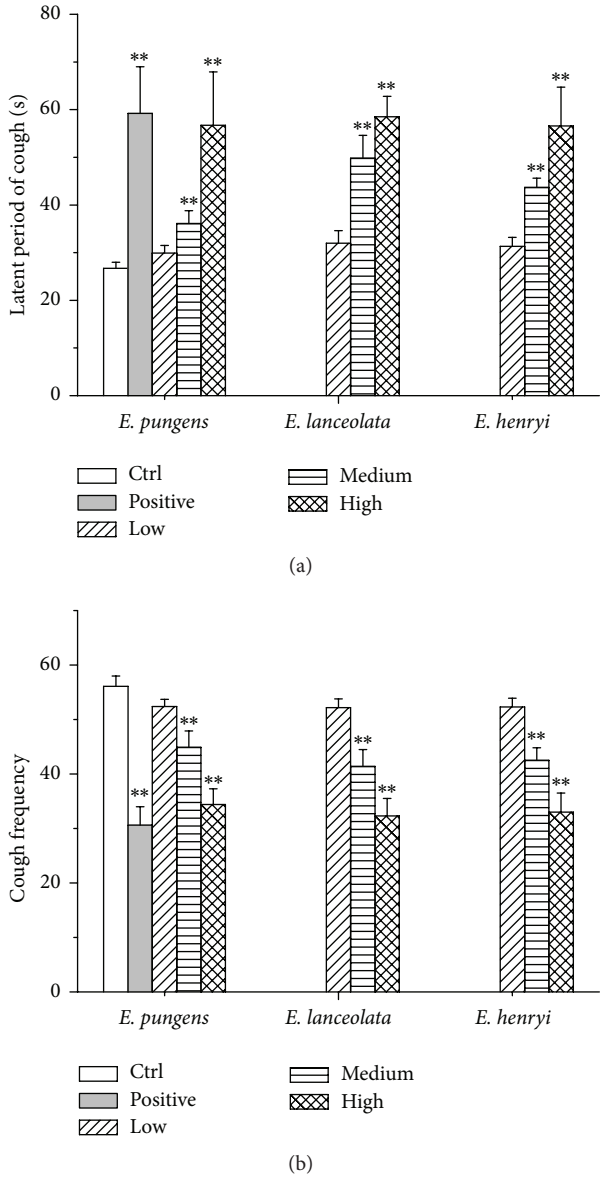

Figure 2: Effect of control, positive (pentoxyverine 50 mg/kg), water extracts of *E. pungens*, *E. lanceolata,* and *E. henryi* leaf (low, medium, and high doses of 4.4, 8.8, and 17.6 expressed as being equal to the weight of crude material per body weight) on the aqueous ammonia-induced latent period of cough (a) and cough frequency (b) after administration for 7 days ($n = 10$). Values are presented as mean ± SD, $^{**}P < 0.01$, compared with control group.

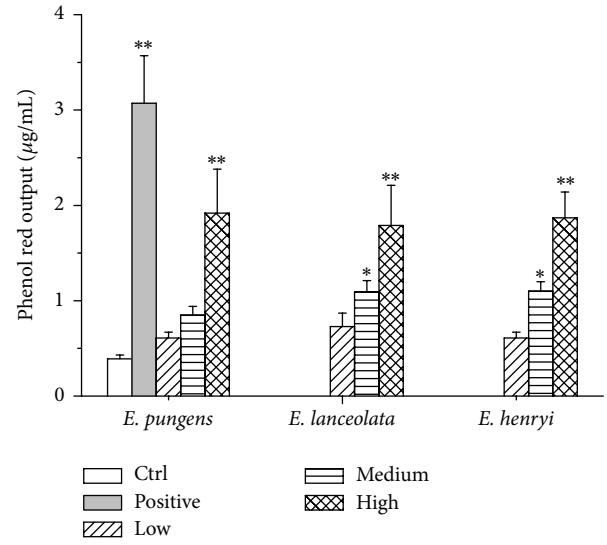

Figure 3: Effect of control, positive (ammonium chloride 1000 mg/kg), water extracts of *E. pungens*, *E. lanceolata,* and *E. henryi* leaf (low, medium, and high doses of 4.4, 8.8, and 17.6 expressed as being equal to the weight of crude material per body weight) on the volume of phenol red in mice's tracheas after administration for 5 days ($n = 10$). Values are presented as mean ± SD, $^{*}P < 0.05$, $^{**}P < 0.01$, compared with control group.

(C3) in the *E. henryi* leaf (Figure 6). Moreover, there was more similarity on overlap peaks between 10 and 40 min (Figure 7) and the peaks belonging to flavonoids compounds and characteristic absorptive wavelengths were also listed in Table 1.

4. Discussions

Elaeagnus pungens leaf has been traditionally used as an antiasthmatic remedy for several hundred years. It is nontoxic under oral administration for a long time in adult without definite IC_{50} value in mice [17]. Through the resource and medical value survey of the *Elaeagnus* plants in Hubei province, Tujia nationality area, and Guizhou and Yunnan provinces, we found that *Elaeagnus henryi* and *Elaeagnus lanceolata* are also generally used as *E. pungens* to treat shortness of breath, cough, or bronchitis. In the present study, water was chosen to extract the three *Elaeagnus* leavesaccording to the previous study suggesting that water fraction is the most active part of *E. pungens* leaf by the pharmacological evaluation [20]. Then, the water extracts of the three *Elaeagnus* leaves were evaluated on the relaxant, antitussive, and expectorant effects *in vivo*.

On the histamine and acetylcholine chloride-induced bronchoconstriction in guinea pigs, the water extracts significantly increased the preconvulsive time in asthma relieving (Figure 1). They also showed significant antitussive effect through the increase of latent period of cough and inhibition of cough (Figure 2). Additionally, the water extracts enhanced phenol red secretion into the airway with ammonium chloride as positive expectorant *in vivo* (Figure 3), which indicated that the expectorant action may be related to its ability to increase tracheobronchial mucus secretion and, thus, may decrease viscosity of mucus [26]. All three water extracts appeared to be the dose-dependent activities and significant differences at the medium and high dosages with comparison to the control group ($P < 0.01$). However, there were no significant differences in the pharmacological actions between the three *Elaeagnus* leaves. It implies that *E. henryi* and *E. lanceolata* had good pharmacological effects on therelaxant, antitussive, and expectorant activities as well as *E. pungens*.

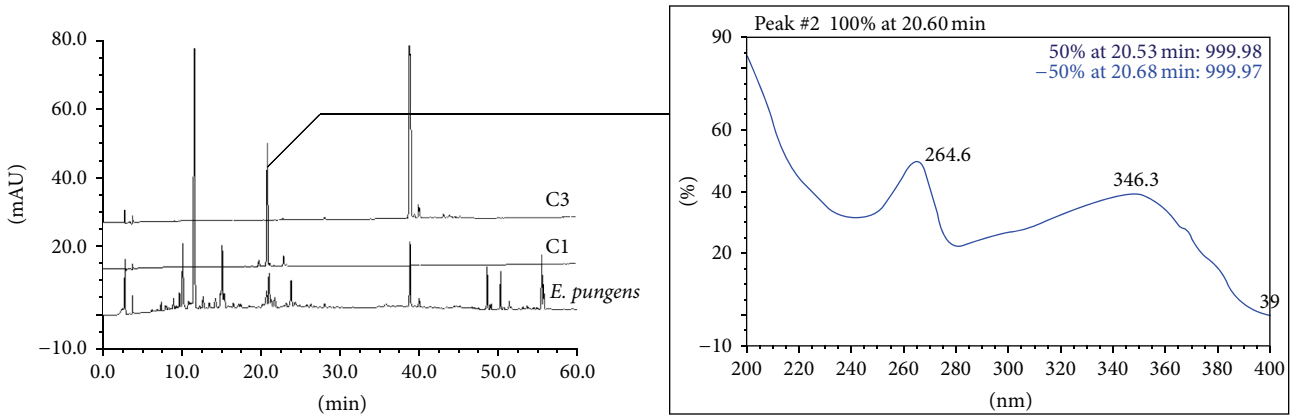

Figure 4: HPLC-DAD chromatographies of *E. pungens* leaf and monomeric compounds C1 (kaempferol 3-*O*-α-rhamnopyranosyl-(1 → 2)[α-rhamnopyran-osyl-(1 → 6)]-β-D-galactopyranoside, t_R = 20.600 min), C3 (kaempferol 3-*O*-(6″-*O*-*E*-*p*-coumaryl)-β-D-glucopyranoside, t_R = 38.763 min).

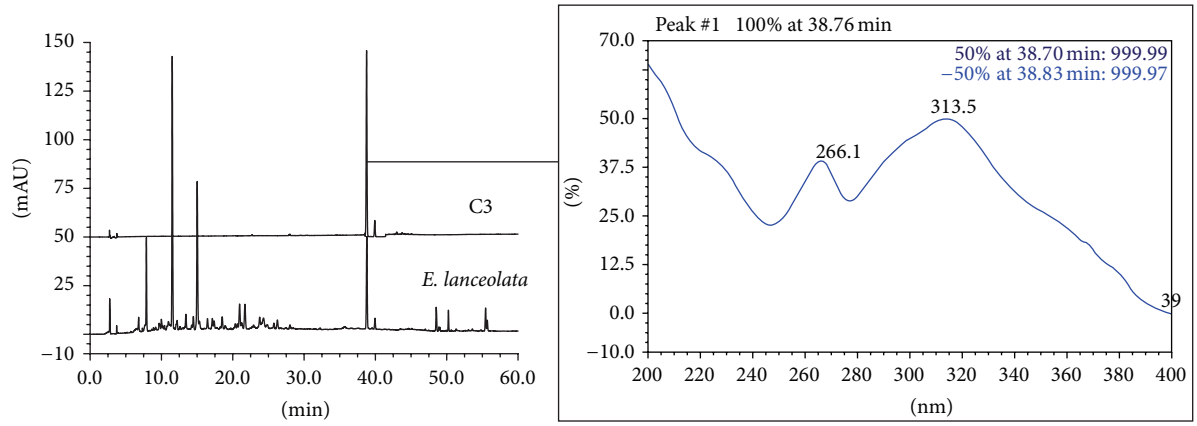

Figure 5: HPLC-DAD chromatographies of *E. lanceolata* leaf and monomeric compounds C3.

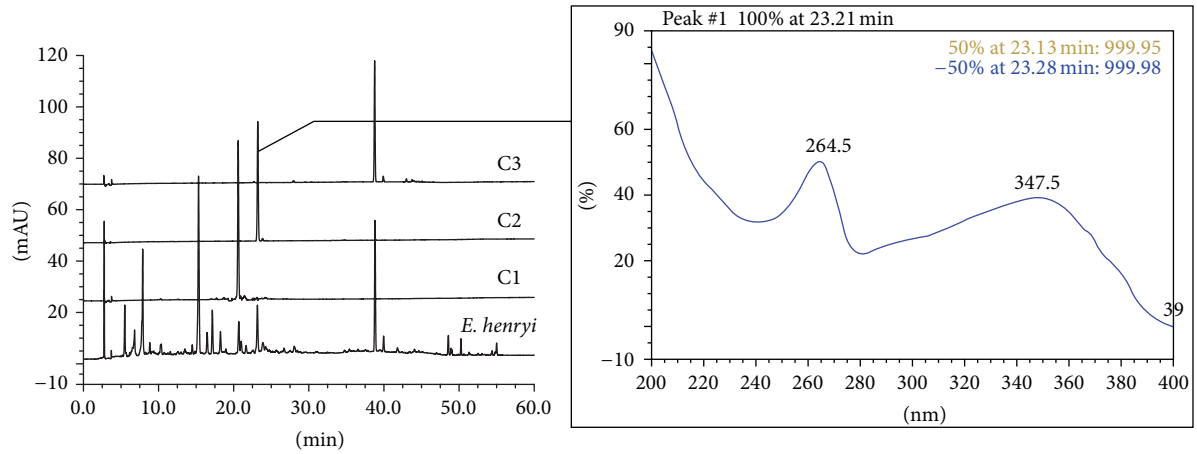

Figure 6: HPLC-DAD chromatographies of *E. henryi* leaf and monomeric compounds C1, C2 (kaempferol 3-*O*-α-L-rhamnopyranosyl-(1 → 2)-β-D-glucopyranoside, t_R = 23.100 min), and C3.

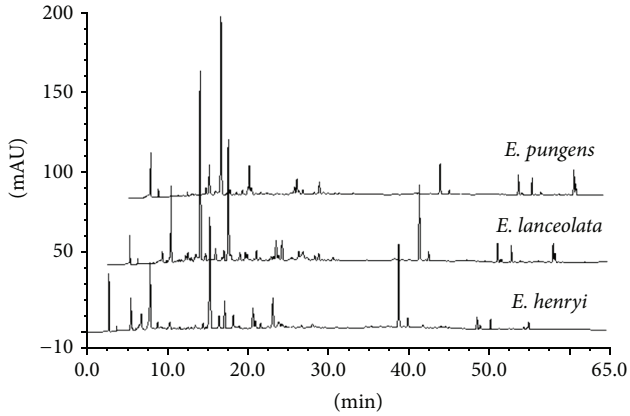

Figure 7: HPLC chromatographies of *E. pungens, E. lanceolata,* and *E. henryi* leaf.

E. pungens leaf is acclaimed to treat asthma and chronic bronchitis induced by weakness of lung Qi in the view of the traditional theory of traditional Chinese medicine. In our previous study, chemical components of *Elaeagnus pungens* leaf are isolated, purified, and identified. The results indicates that it mainly contains many flavonoids of which the chemical structures are characterized by quercetin, kaempferol, and isorhamnetin as aglycones linking with glycosyl groups [18, 19]. Many other researches have verified that flavonoids from Chinese herbs are effective on antiasthmatic, antitussive, and expectorant properties, for example, naringenin from *Exocarpium Citri* Grandis and total flavones from *Acanthopanax senticosus* [26–29]. Moreover, plants from *Elaeagnus* are reported to have flavonoid constituents such as *E. henryi*, *E. lanceolata*, *E. angustifolia,* and *E. bockii* [8–10, 21]. It prompted us to lay a hypothesis that these flavonoids in the three *Elaeagnus* leaves might have the antiasthmatic, antitussive, and expectorant activities. So, we analyzed the chemical components and contrasted flavonoids peaks by HPLC-DAD. The result showed that the three *Elaeagnus* leaves had more similarity on overlap peaks between the 15 and 60 min (Figure 6) and the peaks belonging to flavonoids compounds (Table 1). However, due to the nature of multiple chemical constituents involved in the natural plants as well as the multifactorial condition of asthma, it is very important to further separate chemical constituents from the three *Elaeagnus* leaves being effective on the relief of bronchoconstriction, inhibition of cough, and increase of secretion output. Further studies are necessary to clarify the mechanism by which the three *Elaeagnus* leaves possess the antiasthmatic, antitussive, and expectorant activities.

In conclusion, our study indicated that the water extracts of *Elaeagnus pungens*, *Elaeagnus henryi,* and *Elaeagnus lanceolata* leaf demonstrated the significantly antiasthmatic, antitussive, and expectorant effects *in vivo*. These effects were the important evidence for the traditional use of *E. henryi* leaf and *E. lanceolata* leaf as antiasthmatic remedy. Moreover, there was more similarity on overlap peaks between the 10 and 40 min retention time by HPLC and the peaks belonging to flavonoids compounds, suggesting that the main constituents of the three *Elaeagnus* leaves were flavonoid for the pharmacological activities. It persuaded us to draw a conclusion that *E. henryi* and *E. lanceolata* played an important role as well as *E. pungens* to treat asthma and chronic bronchitis.

Table 1: Flavonoids peaks of *E. pungens*, *E. lanceolata,* and *E. henryi*. leaf by HPLC-DAD.

Samples	Retention time/min	UV λ_{max}/nm
E. pungens	10.780	265, 350
	20.650	264, 347
	20.960	264, 346
	23.117	264, 347
	23.747	264, 347
	26.243	264, 344
	27.997	264, 348
	38.773	266, 313
	39.917	266, 308
E. lanceolata	18.520	255, 353
	20.960	262, 350
	23.777	264, 347
	25.770	240, 318
	26.260	264, 344
	28.020	264, 350
	38.797	266, 313
	39.940	266, 313
E. henryi	15.323	237, 327
	17.160	238, 327
	18.240	255, 352
	20.683	264, 346
	20.997	264, 342
	21.643	255, 355
	23.157	264, 350
	23.897	242, 333
	28.063	264, 355
	38.817	266, 313
	39.963	266, 315
	41.800	240, 325

Conflict of Interests

The authors declare that there is no conflict of interests.

Acknowledgments

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Research Article

Ferulic Acid Induces Th1 Responses by Modulating the Function of Dendritic Cells and Ameliorates Th2-Mediated Allergic Airway Inflammation in Mice

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This study investigated the immunomodulatory effects of ferulic acid (FA) on antigen-presenting dendritic cells (DCs) *in vitro* and its antiallergic effects against ovalbumin- (OVA-) induced Th2-mediated allergic asthma in mice. The activation of FA-treated bone marrow-derived DCs by lipopolysaccharide (LPS) stimulation induced a high level of interleukin- (IL-) 12 but reduced the expression levels of the proinflammatory cytokines IL-1β, IL-6, and tumor necrosis factor- (TNF-) α. Compared to control-treated DCs, FA significantly enhanced the expressions of Notch ligand Delta-like 4 (Dll4), MHC class II, and CD40 molecules by these DCs. Furthermore, these FA-treated DCs enhanced T-cell proliferation and Th1 cell polarization. In animal experiments, oral administration of FA reduced the levels of OVA-specific immunoglobulin E (IgE) and IgG_1 and enhanced IgG_{2a} antibody production in serum. It also ameliorated airway hyperresponsiveness and attenuated eosinophilic pulmonary infiltration in dose-dependent manners. In addition, FA treatment inhibited the production of eotaxin, Th2 cytokines (IL-4, IL-5, and IL-13), and proinflammatory cytokines but promoted the Th1 cytokine interferon- (IFN-) γ production in bronchoalveolar lavage fluid (BALF) and the culture supernatant of spleen cells. These findings suggest that FA exhibits an antiallergic effect via restoring Th1/Th2 imbalance by modulating DCs function in an asthmatic mouse model.

1. Introduction

Asthma is a heterogeneous chronic inflammatory lung disease that is characterized by various airway obstructions, bronchial hyperresponsiveness, and airway inflammation. It is recognized that Th2 cells and their cytokines (interleukin- (IL-) 4, IL-5, and IL-13) are responsible for initiating and maintaining Th2-associated asthma [1]. These Th2 cytokines induce an inflammatory cascade that comprises allergen-specific immunoglobulin (Ig)E production, mast cell activation, eosinophil recruitment, and airway hyperresponsiveness (AHR) [2]. In addition to Th2-cell effects, dendritic cells (DCs), as professional antigen-presenting cells (APCs), play an important role in antigen presentation in the airways, and the expression of costimulatory molecules and cytokine profile by DCs can determine whether T cells differentiate into type 1 T-helper (Th1) cells, Th2 cells, or regulatory T cells (Tregs) [3, 4]. In addition, the ability of DCs to polarize Th2 responses may be enhanced by engagement of Notch receptors at the surface of T cells with ligands Jagged on DCs [5]. Therefore, inhibition of Th2 effector responses by modulating DCs maturation and function is considered a promising immunomodulatory strategy to treat Th2-associated allergic asthma.

Ferulic acid (trans-4-hydroxy-3-methoxycinnamic acid; FA; molecular weight 194.18) belongs to the family of phenolic acids and is widely found in vegetables, fruits, and some beverages such as coffee and beer [6]. Moreover, FA is also

a component of Chinese medicinal herbs, such as *Angelica sinensis*, *Cimicifuga racemosa*, and *Ligusticum chuanxiong*. The daily amount of FA ingested was calculated to be around 150~250 mg [7]. After oral ingestion, FA is quickly absorbed and reaches a peak plasma concentration within 30 min [8]. Because of its antioxidative and anti-inflammatory activities, FA was shown to have therapeutic effects in various diseases such as Alzheimer's disease, cancer, diabetes mellitus, and arthritis [9–12]. Therefore, the abundant dietary sources, the relatively low toxicity, and potential bioactivities of FA may be capable of influencing immune cell functions in allergic immune responses and provide possible alternative options for relieving allergic asthma-associated symptoms. However, to date, the protective effects of FA against chronic inflammatory lung diseases, such as asthma, and the underlying mechanisms are still unavailable.

In the current study, we attempted to clarify whether treatment with FA can alter the phenotype and regulatory ability of DCs on T cells and achieve antiallergic effects to alleviate the development of airway symptoms in a mouse model of allergic asthma. Our data revealed that FA modulates DC function to promote interferon- (IFN-) γ production by activated T cells and convert T cells with Th1 activity in Th2-driven allergic diseases. These findings provide insights into how FA affects the Th2-biased immune response and provide guidance on the use of FA as an antiallergic adjuvant in treating Th2-mediated allergic asthma.

2. Materials and Methods

2.1. Mice. Female BALB/c and C57BL/6 mice were purchased from the National Laboratory Animal Center (Taipei, Taiwan) and maintained in the Animal Center of Taipei Medical University. Animals were housed in group cages (4-5 animals per cage) with free access to food and water. The environment was controlled on a 12 h dark-light cycle at a temperature of $23 \pm 2^{\circ}$C. Animal care and handling protocols were evaluated and approved by the Animal Committee of the College of Medicine, Taipei Medical University (approval number LAC-98-0158).

2.2. Preparation of Bone Marrow-Derived DCs (BMDCs). DCs were obtained by culturing BALB/c bone marrow cells in RPMI-1640 containing 5% fetal bovine serum (FBS), glutamine, penicillin/streptomycin, murine IL-4 (1000 U/mL), and GM-CSF (500 U/mL) for 6 days. Nonadherent cells were harvested and their purity was identified by flow cytometry, gated on $CD11c^+$ cells. The FACS analysis showed that there were 70%~80% of DCs in this cell population.

2.3. Determination of Cytokine and Chemokine Levels. On day 6, 10^6 cells/mL of BMDCs were stimulated with lipopolysaccharide (LPS; 1 μg/mL) plus various concentrations of FA (20, 100, and 400 μM) which was purchased from Sigma-Aldrich (St. Louis, MO, USA) or LPS alone for 24 h to achieve activation before use in the cytokine or phenotypical assays. In the experiments, untreated DCs were cultured with medium alone as the control. Concentrations of IL-1β, IL-6, IL-10, IL-12, and tumor necrosis factor- (TNF-) α in culture supernatants were evaluated by enzyme-linked immunosorbent assay (ELISA) kits (IL-1β, IL-6, IL-12, and TNF-α from eBioscience, San Diego, CA, USA; IL-10 from Duoset, R&D Systems, Minneapolis, MN, USA). The concentration of cytokines was measured by converting the OD values of the samples to pg/mL values from the standard curve.

The levels of eotaxin, IL-1β, IL-4, IL-5, IL-6, IL-10, IL-13, TNF-α, and IFN-γ in bronchoalveolar lavage fluid (BALF) and culture supernatants of splenocytes were also determined by commercial ELISA kits (eotaxin, IL-4, IL-5, IL-13, and IFN-γ from Duoset, R&D Systems).

2.4. Quantitative Real-Time Polymerase Chain Reaction. On day 6 of culture, BMDCs were collected and 10^6 cells/mL were treated with LPS (1 μg/mL) and various concentrations of FA (20, 100, and 400 μM) or LPS alone for 6 h. Untreated DCs were cultured with medium alone as a control. After incubation, cells were collected, and total RNA was isolated according to the TRIzol method (Sigma-Aldrich, St. Louis, MO, USA). Complementary (c)DNA was synthesized from total RNA using the cDNA RT kit (Applied Biosystems, Foster City, CA, USA). Real-time PCR was performed with cDNA using the PCR master mix and TaqMan assays (Applied Biosystems). qPCR detection of mouse GAPDH, Delta1, Delta4, Jagged1, and Jagged2 was conducted in triplicate using an Applied Biosystems 7900 PCR system. Quantification was carried out using GAPDH gene expression as an internal normalization control, and the fold change in transcription was calculated using the comparative threshold cycle (C_T) method, $2^{-\Delta\Delta C_T}$.

2.5. Flow Cytometric Analysis. Cytometric analysis was performed with a BD FACSort cell analyzer and CellQuest (BD Biosciences) software. DCs were stained with antibodies in phosphate-buffered saline (PBS) for 30 min at 4°C and washed with 2 mL of PBS. Then, cells were suspended in 0.5 mL of PBS with 0.1% sodium azide. Staining with isotype control antibodies was performed in all experiments. DCs were gated according to the standard forward- and side-scatter profiles of $CD11c^+$ large cells. Anti-CD11c-fluorescein isothiocyanate (FITC), anti-CD40-FITC, anti-CD86-FITC, anti-CD11c-phycoerythrin (PE), anti-I-A/I-E (MHC class II)-PE, anti-CD80-PE, and anti-Jagged1-PE monoclonal antibodies (mAbs) were purchased from eBioscience; anti-Delta1-PE, anti-Delta4-PE, and anti-Jagged2-PE mAbs were purchased from BioLegend.

2.6. Mixed Lymphocyte Reaction (MLR). This assay can be used to evaluate the stimulatory ability of DCs to alter T-cell proliferation and cytokine production. Approximately 10^6 day-6 BMDCs derived from BALB/c mice were treated with LPS (1 μg/mL), LPS plus FA (400 μM), or medium only. After 24 h of culture, DCs were irradiated with 3000 rad (^{137}Cs source). Allogeneic naïve $CD4^+$ T cells from female C57BL/6 mice were purified by using $CD4^+$ T-cell isolation kit (Miltenyi Biotec, Auburn, CA, USA). Then $CD4^+$ T cells (2×10^5 cells/well) were cultured with different proportions of irradiated DCs (0.5×10^4, 10^4, 2×10^4, and 2×10^4 cells/well) in 96-well round-bottom plates for 2 days. Tritiated

thymidine (1 μCi/well, New England Nuclear, Boston, MA, USA) incorporation for 16 hours was determined with a dry scintillation counter (Packard Instrument, Meriden, CT, USA). Additionally, levels of IL-5 and IFN-γ production in the culture supernatant were assayed by ELISA kits.

2.7. Experimental Protocol for the Th2-Cell-Mediated Allergic Asthma Model. Female BALB/c mice at 6 weeks of age (weight range 19~20 g) were intraperitoneally (i.p.) sensitized with 50 μg OVA (grade V; Sigma-Aldrich, St. Louis, MO) plus 2 mg of aluminum hydroxide (Thermo Scientific, Rockford, IL, USA) on day 1 and boosted with 25 μg OVA in the same dosage of adjuvant on days 14 and 28. Subsequently, mice were intranasally (i.n.) challenged with 100 μg OVA on days 43 and 44. Finally, mice were exposed to an OVA aerosol challenge on days 45, 46, and 47 by inhalation 5% OVA in a normal saline using an atomizer (DeVilbiss Health Care, Somerset, PA, USA) for 30 min, and AHR was measured 1 d after the final challenge (on day 48). To examine the effects of various dosages (25, 50, and 100 mg/kg of body weight) of FA, three groups of mice (FA (25), FA (50), and FA (100)) were, respectively, orally fed three doses of FA on days 21 to 47. OVA-sensitized and OVA-challenged mice were fed sterilized water instead of FA as a positive control (PC). Negative control (NC) mice were injected with PBS and challenged with OVA. During the experiment, no abnormal symptoms or toxic effects were observed in these groups. Serum samples were collected on days 1, 7, 28, 35, and 48.

2.8. Serum Antibody Assay. OVA-specific IgE, IgG_1, and IgG_{2a} antibody titers in sera were determined by ELISA (Becton Dickinson Biosciences, San Jose, CA, USA) as described previously [13]. In brief, 96-well microtiter plates were coated with OVA (10 μg/mL) in $NaHCO_3$ buffer (pH 9.6) at 4°C overnight, and then treated with mouse sera followed by biotin-conjugated anti-mouse IgE, IgG_1, or IgG_{2a} (PharMingen, San Diego, CA) for 1 h at 37°C. Then, avidin-horseradish peroxidase (1 : 5000, Pierce Biotechnology, Rockford, IL, USA) was added to each well for another 30 min at room temperature. Finally, the reaction was developed by 2,2′-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (Sigma-Aldrich, St. Louis, MO) and the absorbance was determined at 405 nm. The levels of antibody were compared with OVA-specific IgE, IgG_1 and IgG_{2a} standard serum with predetermined concentrations (immunoglobulin concentrations: IgE = 1.5 μg/mL, IgG_1 = 32.5 μg/mL IgG_{2a} = 15.2 μg/mL). The concentration of standard serum was arbitrarily assigned as 1 ELISA unit (1 EU).

2.9. Measurement of Airway Responsiveness. At 24 h after the final aerosol OVA exposure, *in vivo* lung function was measured by detecting changes in airway resistance in response to increasing doses of aerosolized methacholine (MCh, Sigma-Aldrich) in anesthetized mice as described previously [14]. MCh aerosol was generated with a nebulizer and administrated directly through the ventilator for 3 minutes. Airway reactivity was then monitored and data were expressed as the pulmonary resistance (R_L) to estimate airway resistance.

2.10. Analysis of the Cellular Composition of BALF. After measuring the pulmonary function parameters, lungs of mice were lavaged three times with 1 mL of HBSS and the lavage fluids were centrifuged. The BALF supernatants were further assayed by the ELISA method, and cell pellets were resuspended in 1 mL HBSS. Total cells counts and differential counts of cytospin preparations stained by Liu's stain solution (Chi I Pao, Taipei, Taiwan) were determined by microscopy. At least 200 cells were counted and identified as macrophages, eosinophils, neutrophils, and lymphocytes under ×200 magnification. Because macrophages and lymphocytes have a similar staining profile, the different cell types were identified based on their morphology and size. The lymphocyte has a round nucleus and a high nuclear to cytoplasmic (N : C) ratio. Macrophages have a relatively low N : C ratio, and the cell size is larger (18–30 μm in diameter) than lymphocytes (6 μm in diameter).

2.11. Histological Examination of Lung Tissues. After being lavaged, the lungs were immediately inflated with 10% formalin, fixed for 24 hours and embedded in paraffin. For histological examination, 5 μm-thick sections were cut, placed on glass slides, deparaffinized, and sequentially stained with hematoxylin-eosin (H&E) to evaluate inflammatory cell infiltration, or with periodic acid-Schiff (PAS) to identify goblet cells. Light microscopy was used for the histopathological assessment.

2.12. Statistical Analysis. Experimental results are presented as the mean ± standard error of the mean (SEM). Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Dunnett's post hoc test. A p value of less than 0.05 was considered to be significant.

3. Results

3.1. FA Treatment Enhanced Th1-Polarizing Cytokine Production and Reduced Proinflammatory Cytokine Secretion by LPS-Stimulated DCs. Before testing the effects of FA on BMDCs, we examined the effect of FA on cell viability. After 72 h of treatment with different doses of FA (0~5000 μM) in BMDCs, cell numbers were counted and cell viability was determined by using a MTT assay. Results showed that the highest concentration (400 μM) of FA used in the following experiments was not cytotoxic (Figure S1 in Supplementary Material available online at http://dx.doi.org/10.1155/2015/678487). We then addressed the impact of FA on the production of cytokines by DCs. LPS has been described as an inducer of DC activation and maturation. We used LPS as a positive control in this study. When BMDCs were treated with FA alone, there was no effect on cytokine production (unpublished data). Then, BMDCs were exposed to FA (20, 100, and 400 μM) following LPS (1 μg/mL) stimulation. In the presence of FA, dose-dependent increased levels of the Th1-polarizing cytokine IL-12 were observed in LPS-stimulated DCs (Figure 1). However, a dose-dependent decrease in IL-10 production was detected in DCs treated with FA plus LPS. We then examined the impact of FA on the production of proinflammatory cytokines, and as depicted in Figure 1, FA

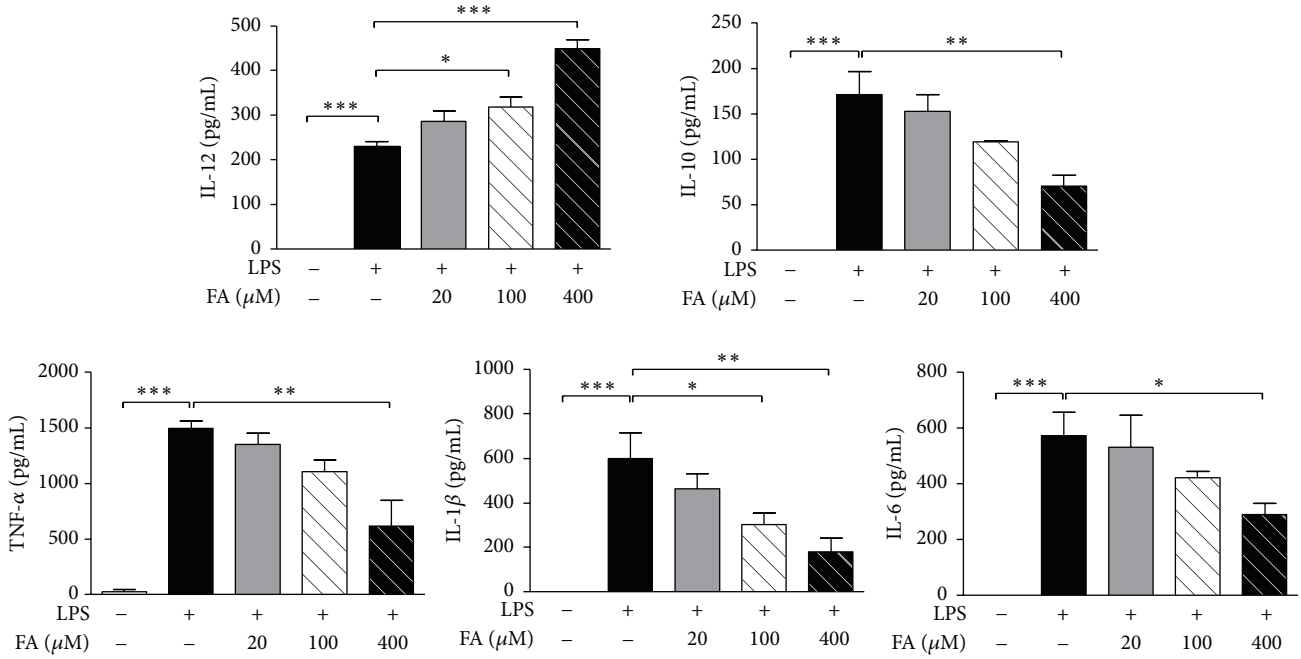


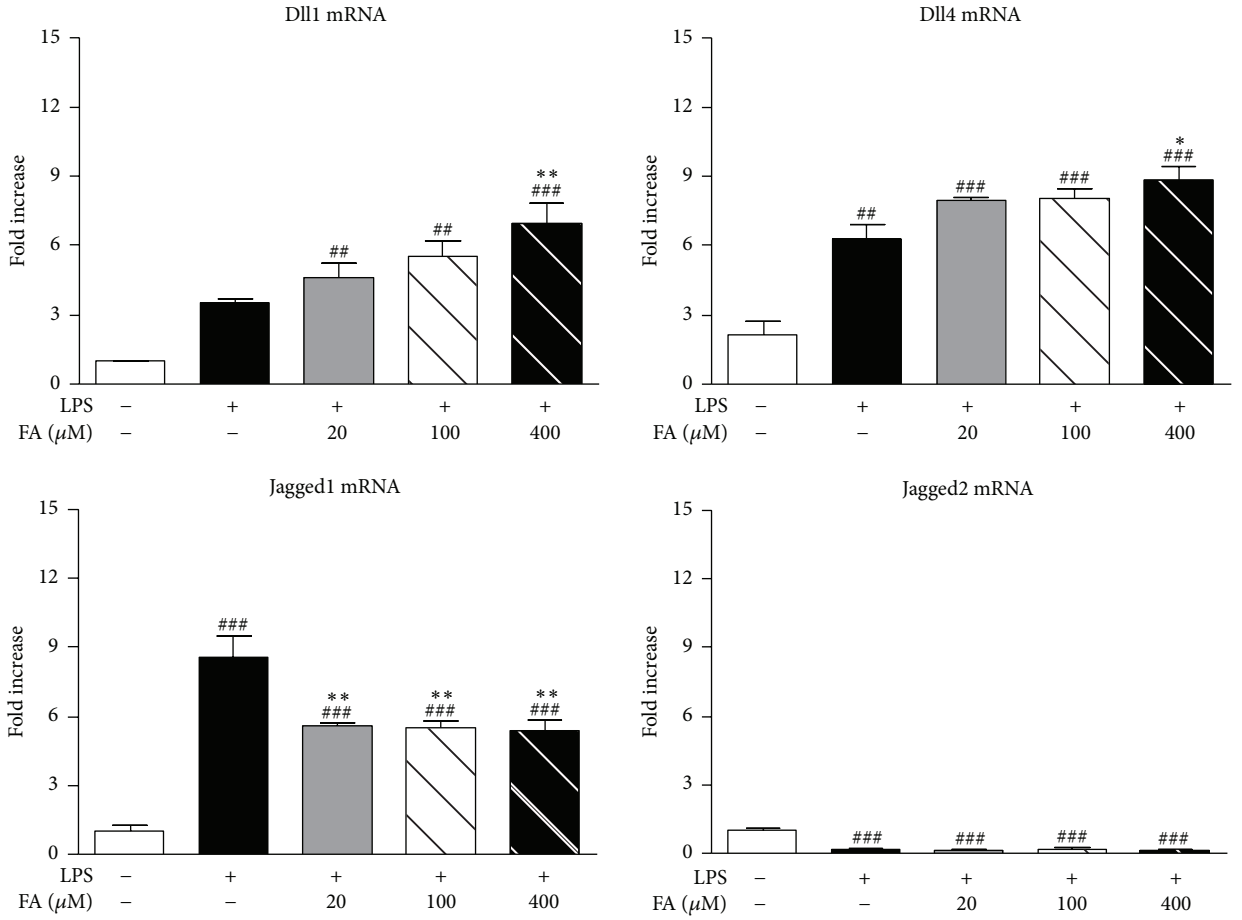
Figure 1: Ferulic acid modulates cytokine secretion in lipopolysaccharide-stimulated dendritic cells. On day 6 of culture, bone marrow-derived dendritic cells (BMDCs) were collected and treated with RPMI medium 1640, lipopolysaccharide (LPS, 1 μg/mL) alone, or LPS plus various concentrations of FA (20, 100, and 400 μM) for 24 h. Culture supernatants were collected, and cytokine profiles of DCs were measured by an ELISA. Results from three independent experiments are shown, and results are expressed as the mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ versus LPS-treated DCs.

obviously inhibited TNF-α, IL-β, and IL-6 release by LPS-stimulated DCs.

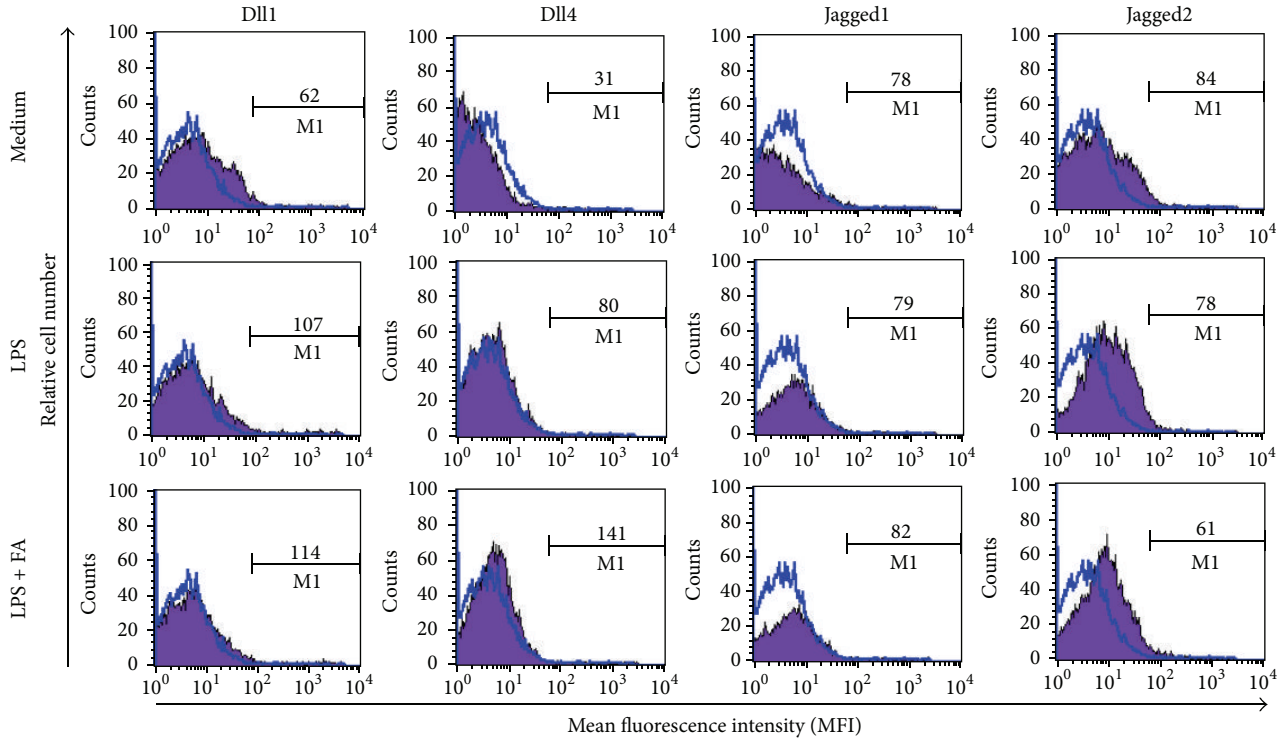
3.2. FA Enhanced Notch Ligand Delta Expression by LPS-Stimulated DCs. Variations in expressions of the Notch ligands, Delta (Delta-like 1 [Dll1] and Delta-like 4 [Dll4]) and Jagged families (Jagged1 and Jagged2), by mature DCs were shown to be crucial for T-cell differentiation. It was established that the Dll group directs T-cell polarization toward Th1, whereas the Jagged group promotes Th2 or Treg responses [15–18]. Thus, we investigated whether FA treatment cooperated with or interfered with their expressions, as assessed using a real-time RT-PCR. After LPS stimulation, DCs expressed higher levels of Dll1, Dll4, and Jagged1 mRNA synthesis compared to untreated DCs (Figure 2(a)). Additionally, compared to LPS-stimulated DCs, LPS combined with FA (400 μM) treatment induced higher amounts of Dll1 and Dll4 transcripts in DCs. In contrast, exposure of DCs to LPS plus FA stimulation significantly reduced Jagged1 mRNA synthesis, although there was no interruption in Jagged2 transcripts. We further examined the surface expressions of these Notch ligands on DCs by performing FACS analyses. Compared to untreated DCs (medium group), a higher level of Dll1 was detected in LPS/FA-stimulated DCs (LPS+FA group), although there was no significant difference between the groups (Figures 2(b) and 2(c)). Notably, our data showed that the pattern of protein expression of Dll4 was significantly correlated with mRNA expression. Expressions of Jagged1 and Jagged2 showed no difference among the groups.

3.3. Effects of FA on the Maturation and Activation of LPS-Stimulated DCs. To investigate whether FA modulated the maturation and activation of mouse BMDCs *in vitro*, we compared the phenotype of mouse DCs treated with medium, LPS alone, or LPS plus FA (400 μM) for 24 h. As shown in Figures 3(a) and 3(b), expressions of MHC class II and CD40 molecules during maturation were markedly upregulated in LPS/FA-treated DCs compared to LPS-treated DCs or untreated DCs, respectively (Figure 3). Furthermore, expressions of CD80 and CD86 molecules in LPS/FA-treated DCs were higher than those of untreated DCs, although the difference was not significant.

3.4. FA- and LPS-Treated DCs Enhanced T-Cell Proliferation and Th1 Polarization. Mature DCs have the capacity to induce proliferation of activated T cells at much higher levels than immature DCs. To determine the ability of DCs to induce T-cell proliferation and cytokine production, an allogeneic MLR assay was used. DCs from BALB/c mice were treated with medium, LPS alone, or LPS plus FA and then cocultured with C57BL/6 mouse-derived naïve $CD4^+$ T cells at various ratios (DC/T-cell ratios of 1 : 5, 1 : 10, 1 : 20, and 1 : 40) *in vitro*. As shown in Figure 4(a), treatment with FA of DCs cultured in the presence of LPS stimulated T-cell proliferative responses more effectively than those of LPS-treated DCs (Figure 4(a)). Additionally, we found that FA modulated the secretion of IFN-γ and IL-5 by activated $CD4^+$ T cells. FA- and LPS-treated DCs markedly enhanced T-cell secretion of IFN-γ in the supernatant compared to LPS-treated

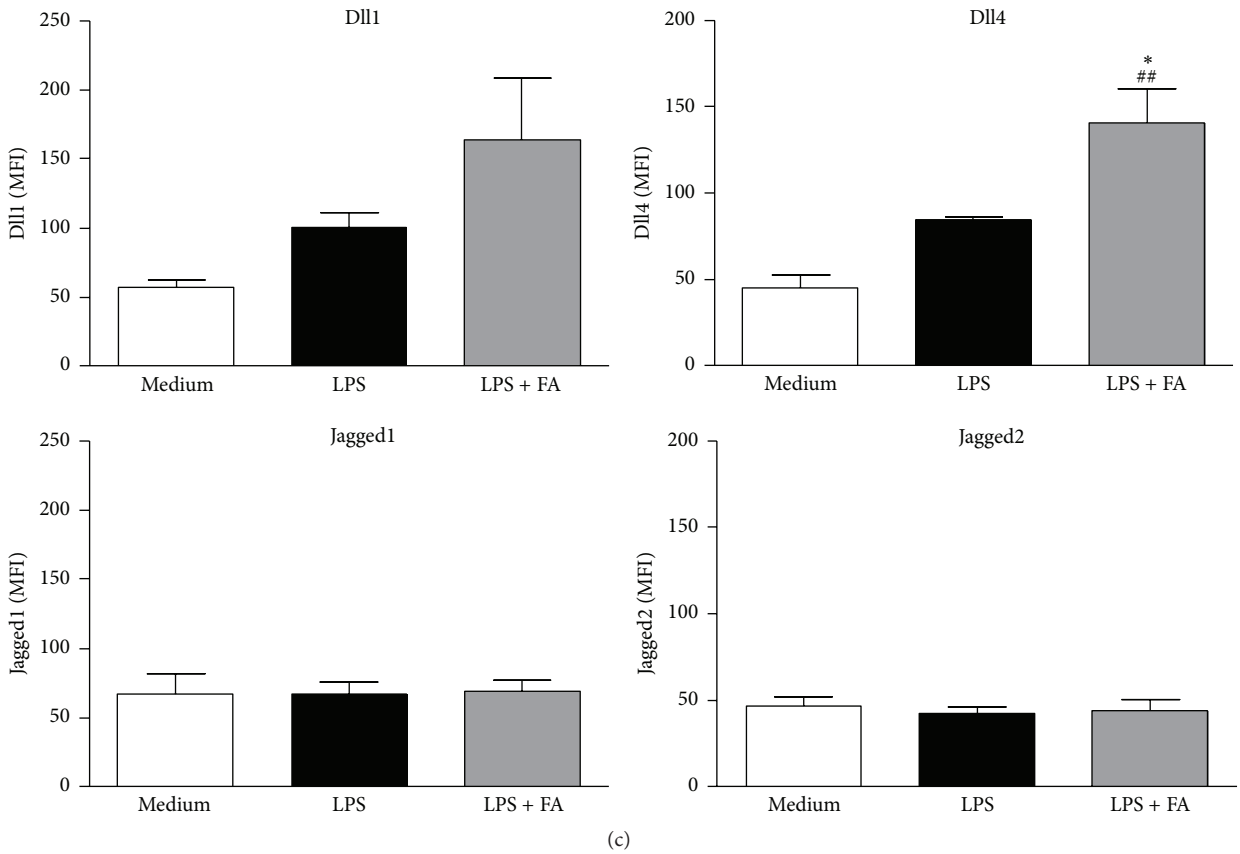


(a)



(b)

Figure 2: Continued.



(c)

Figure 2: Effects of ferulic acid on expressions of Notch ligands in lipopolysaccharide-stimulated dendritic cells. (a) On day 6 of culture, BMDCs were left untreated or treated with LPS (1 μg/mL) alone or LPS plus various concentrations of FA (20, 100, and 400 μM). Untreated immature DCs served as the medium group. Cells were harvested after 5 h, and Jagged1, Jagged2, Dll1, and Dll4 mRNA accumulations were measured using a real-time RT-PCR. Results from three independent experiments are shown, and results are expressed as the mean ± SEM. $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ versus medium-treated DCs. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ versus LPS-treated DCs. (b) Medium-treated DCs, LPS-treated DCs, and FA (400 μM) plus LPS-treated DCs were cultured for 24 h. Then cells were harvested, and surface Notch ligand expressions were analyzed by flow cytometry. Each histogram shown is from one representative experiment performed. Values shown in the flow cytometric profiles are the mean fluorescence intensity (MFI) indexes. Cells were gated on CD11c, and the incidence of $CD11c^+$ cells expressing the Notch ligands is indicated within each histogram. (c) The quantification of surface Notch ligand expressions is analyzed by flow cytometry. Results from three independent experiments are shown and are expressed as the mean ± SEM. $^{\#\#}p < 0.01$ versus medium-treated DCs. $^{*}p < 0.05$ versus LPS-treated DCs.

DCs (Figure 4(b)). In contrast, FA plus LPS-treated DCs significantly suppressed IL-5 release by T cells in a dose-dependent manner. These results showed that FA can enhance the Th1 response through DCs.

We also investigated whether FA could directly affect the cytokines production by T cells. At first, we evaluated the possible cytotoxic effect of FA on $CD4^+$ T cells. After treatment with 1 to 50 μM of FA for 72 h, T cells did not show any change on cell viability as detected by MTT assay (Figure S2A). To analyze the direct effect of FA on T cells, $CD4^+$ T cells were treated with FA (5, 10, and 50 μM) and activated by anti-CD3/anti-CD28 antibodies. Compared to control T cells (anti-CD3/anti-CD28 treatment group), activated T cells cultured in the presence of FA did not show any apparent differences in cytokine production (IFN-γ, IL-10, and IL-5) (Figure S2B). These results indicated that FA could not directly inhibit or promote cytokine production from T cells.

3.5. FA Reduced Serum Anti-OVA IgE and IgG_1 and Enhanced Anti-OVA IgG_{2a} Levels in an Animal Model of Asthma. To investigate the *in vivo* impacts of FA on regulating immune responses of allergic diseases, groups of mice were exposed to OVA sensitization and challenge (Figure 5(a)). These OVA-sensitized mice were orally fed different doses (25, 50, and 100 mg/kg of body weight (BW)) of FA (FA (25), FA (50), and FA (100), resp.). PC mice received distilled water instead of FA. NC mice were neither sensitized with OVA nor administered FA treatment, although they received an OVA challenge. Serum samples were collected on indicated days, and OVA-specific IgE, IgG_1, and IgG_{2a} serum antibody titers were determined by an ELISA. In mice, the Th2 cytokine, IL-4, promotes IgG_1 and IgE production. However, the Th1 cytokine, IFN-γ, induces the production of IgG_{2a}. Therefore, expression levels of subclasses (IgG_1 and IgG_{2a}) or classes (IgE) of antibodies can be used as markers to identify

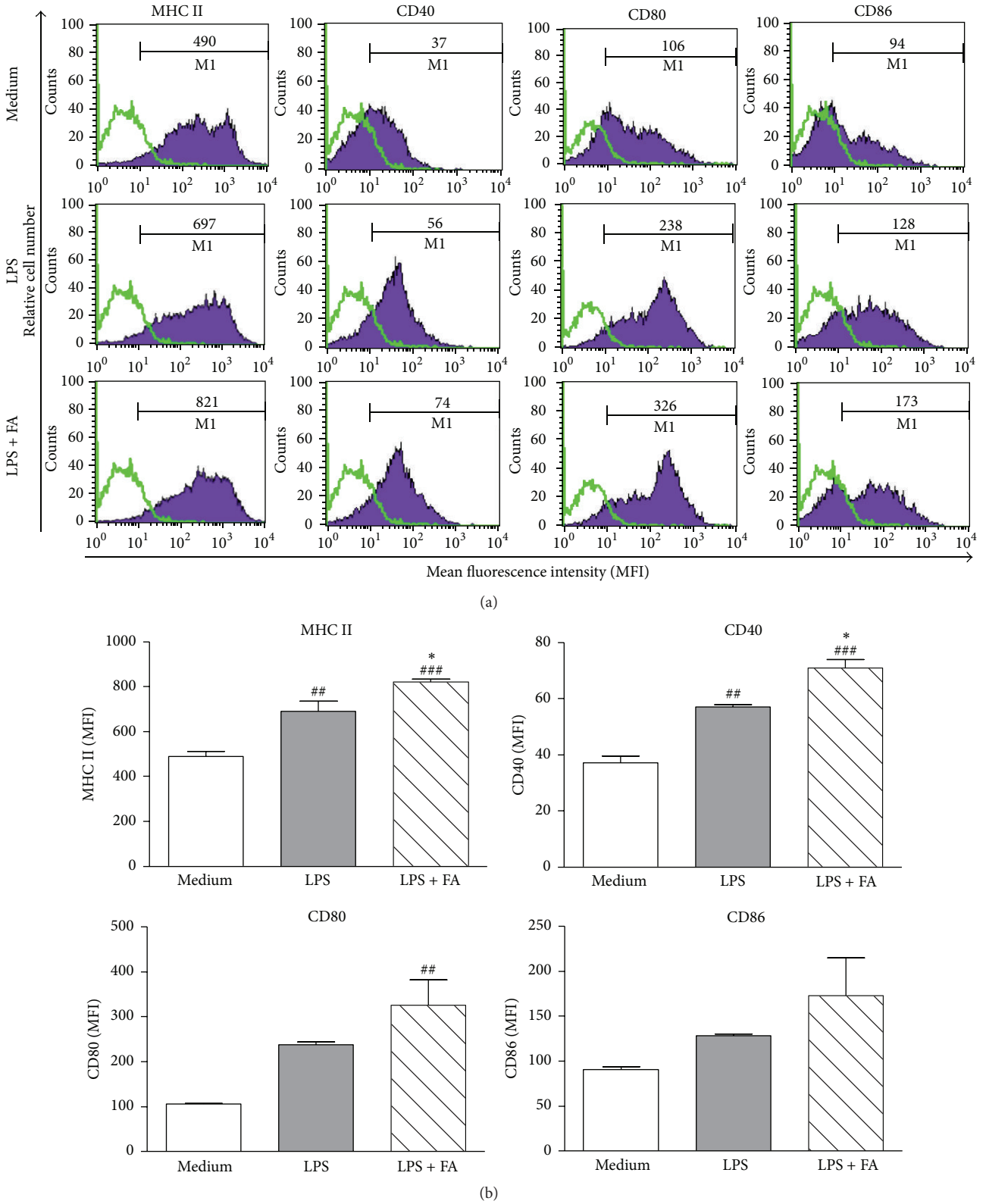


Figure 3: Ferulic acid treatment affects the maturation phenotype of lipopolysaccharide-stimulated dendritic cells. DCs were treated with medium, LPS (1 μg/mL) alone or LPS plus FA (400 μM) for 24 h. After incubation, cells were collected, and expressions of MHC class II, CD40, CD80, and CD86 by DCs were analyzed using flow cytometry. (a) Values shown in the flow cytometric profiles are the mean fluorescence intensity (MFI). Cells were gated on CD11c, and the incidence of $CD11c^+$ cells expressing the surface marker is indicated within each histogram. Values shown are from one representative experiment of three independent experiments performed. (b) The MFI was calculated, and results are expressed as the mean ± SEM from three independent experiments. $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ versus medium-treated DCs. $^{*}p < 0.05$, $^{***}p < 0.001$ versus LPS-treated DCs.

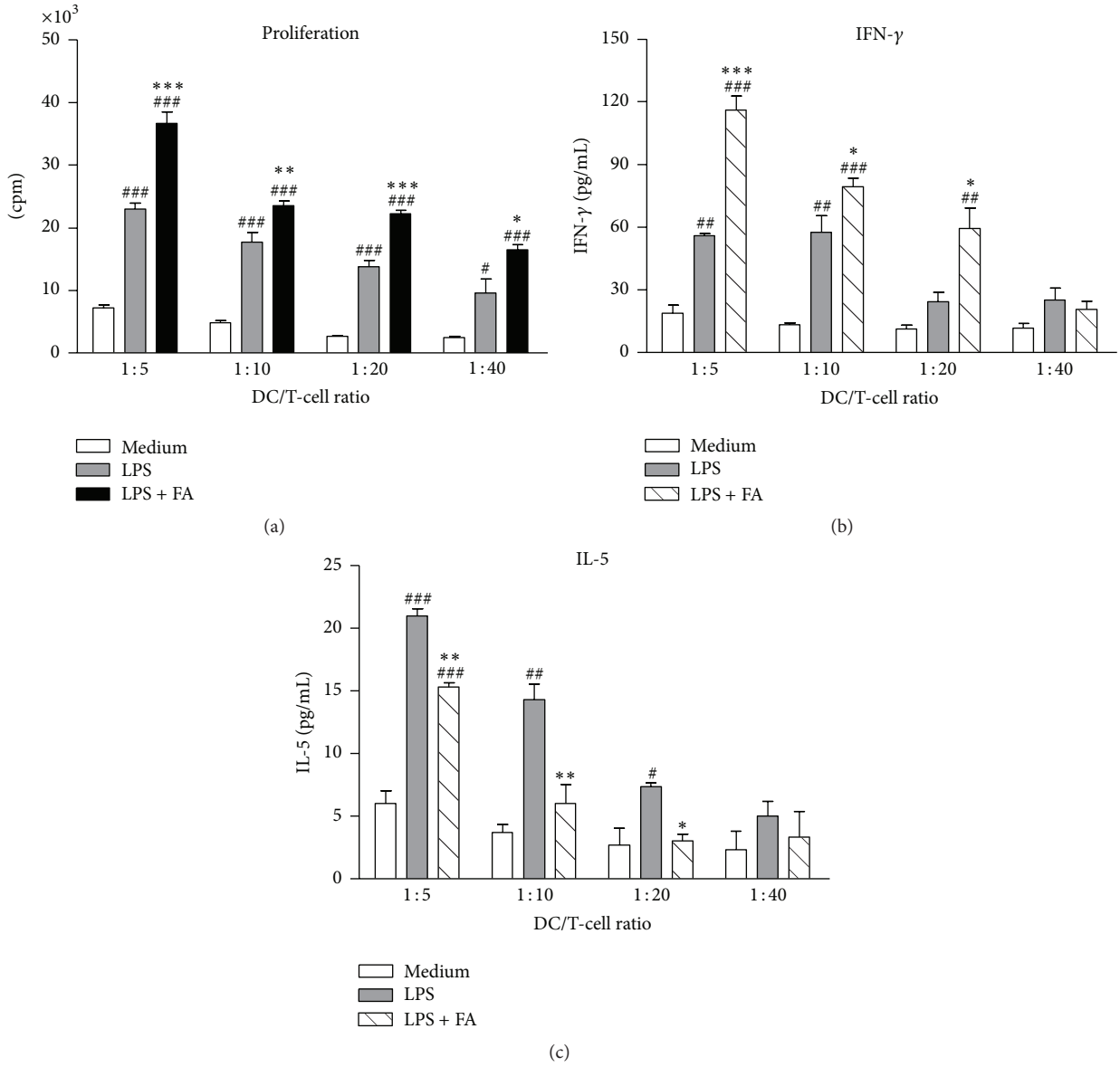


Figure 4: Ferulic acid-modulated dendritic cells enhanced T-cell proliferation and Th1 cell polarization. (a) Modulatory effects of FA on T-cell proliferation. DCs were treated with medium, LPS (1 μg/mL), or LPS plus FA (400 μM) for 24 h. Allogeneic naïve $CD4^+$ T cells (2×10^5 cells/well) were cocultured with different proportions of γ-irradiated immature DCs, LPS-pulsed DCs, or LPS plus FA-treated DCs at DC/T-cell ratios of 1 : 5, 1 : 10, 1 : 20, and 1 : 40 in 96-well round-bottomed plates. After 2 days of culture, cells were pulsed with 1 μCi/well of $[^3H]$-thymidine for 16~18 h. Specific incorporation of $[^3H]$-thymidine was determined with a β-counter, and results are expressed as counts per minute (cpm). (b) Modulatory effects of FA on T-cell cytokine secretion. Concentrations of IFN-γ and IL-5 secreted by naïve $CD4^+$ T cells, which were cocultured with γ-irradiated DCs in 96-well plates for 2 days, were determined by an ELISA. Results in all panels are expressed as the mean ± SEM of three independent experiments. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, and $^{\#\#\#}p < 0.001$ versus medium-treated DCs. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ versus LPS-treated DCs.

what type of Th response was induced in these mice. In the PC group, strong IgE and IgG_1 production and low IgG_{2a} expression were induced (Figure 5(b)). In contrast, FA treatment efficiently inhibited OVA-specific IgE and IgG_1 production. Notably, the administration of FA markedly upregulated IgG_{2a} synthesis compared to the PC group.

3.6. FA Decreased the Severity of AHR and Airway Inflammation. To assess the antiallergic effects of FA on allergic asthma, both the AHR and accumulation of inflammatory cells in BALF were investigated. One day after the last OVA challenge, the airway responsiveness to aerosolized MCh of each group of mice was measured. PC mice, which were administered distilled water instead of FA and sensitized and challenged with OVA, developed markedly increased airway responsiveness to MCh inhalation compared to that of nonallergic NC mice (Figure 6(a)). However, oral treatment with FA dose dependently alleviated the development of AHR

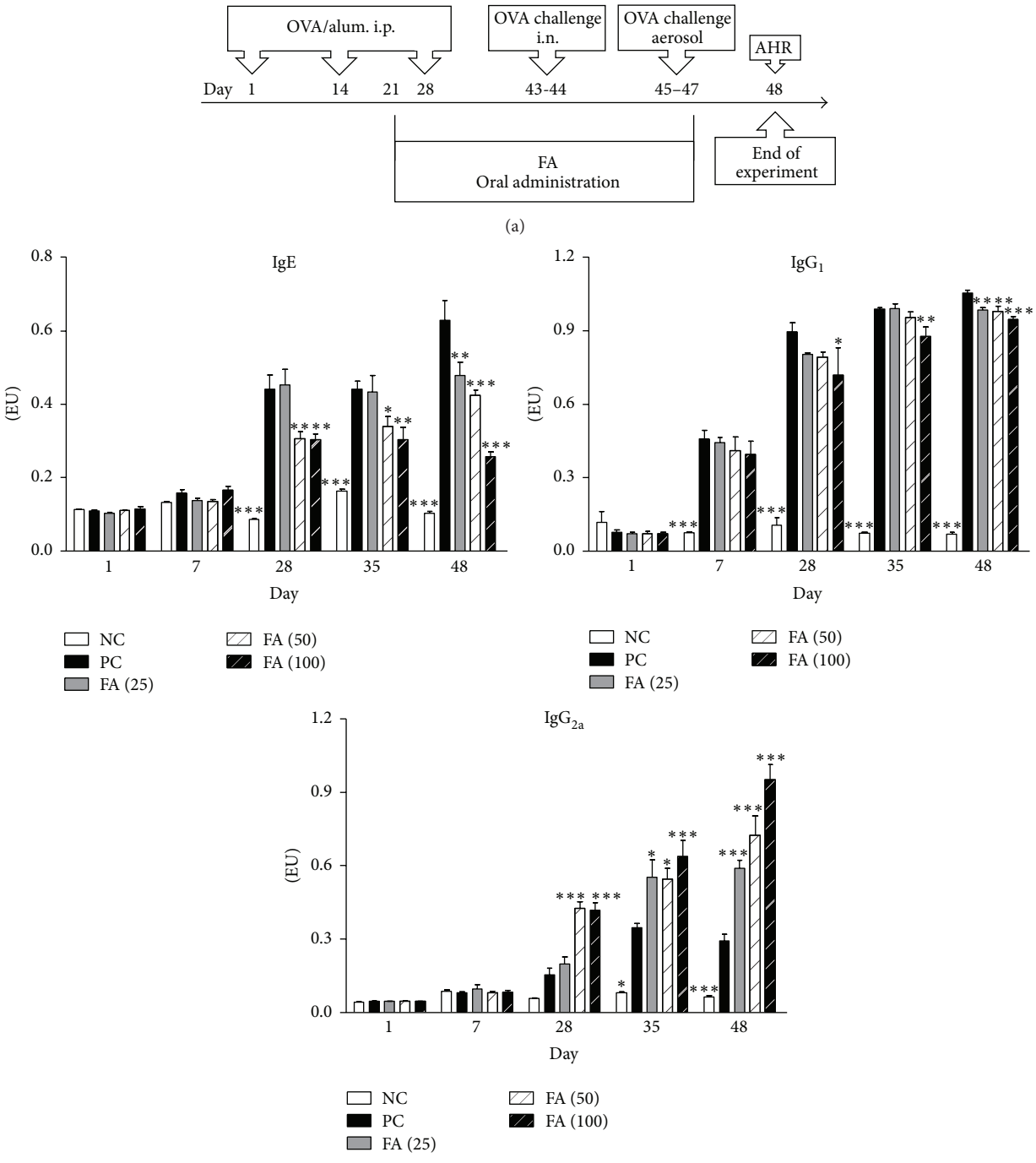


Figure 5: Anti-OVA antibody expression levels of ferulic acid-treated OVA-induced allergic asthmatic mice. (a) Brief description of the protocol of animal sensitization and challenge. On days 1, 14, and 28, all groups of mice were sensitized by an intraperitoneal injection of the OVA allergen. Three groups of mice (FA (25), FA (50), and FA (100)) were, respectively, orally fed 25, 50, and 100 mg/kg of FA on days 21 to 47. Positive control (PC) mice were administered sterilized water instead of FA. Then, mice were intranasally challenged with OVA on days 43 and 44. Subsequently, mice were exposed to OVA aerosols for 3 consecutive days, and AHR was measured 1 day after the last challenge. BALF was collected after measuring the AHR. Negative control (NC) mice were neither sensitized with OVA nor administered FA treatment but were challenged with OVA. (b) IgE, IgG_1, and IgG_{2a} anti-OVA antibody expressions of all groups of mice were measured by an ELISA. Results are expressed as the mean ± SEM ($n = 8$ in each group). $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ versus the PC group.

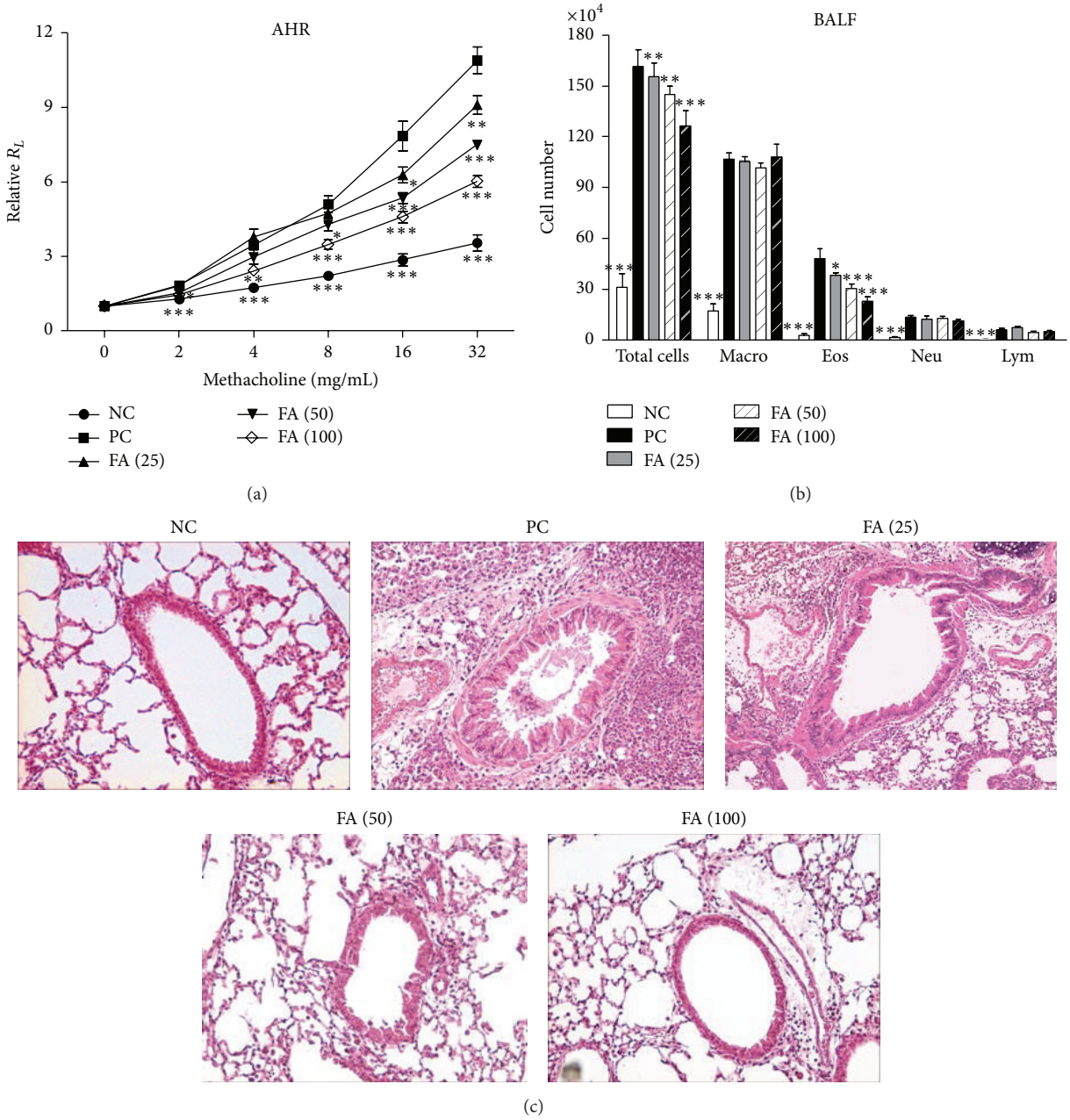


FIGURE 6: Effects of ferulic acid on the airway hyperresponsiveness and airway inflammation. (a) Oral administration of FA suppressed the development of AHR in OVA-sensitized mice. One day after the last OVA challenge, airway resistance was measured in response to increasing concentrations of methacholine (0~32 mg/mL) by invasive body plethysmography. Results are expressed as the mean ± SEM ($n = 8$ in each group) of the pulmonary resistance (R_L) after PBS nebulization. (b) Changes in the cellular composition of the BALF of mice exposed to an aerosolized allergen. One day after measuring the pulmonary function parameters, each group of mice was sacrificed, and the BALF was collected. Cells were counted and classified as macrophages (Macro), eosinophils (Eos), neutrophils (Neu), and lymphocytes (Lym). Results are expressed as the mean ± SEM ($n = 8$ in each group). (c) Effect of FA on lung inflammatory cell infiltration in OVA-induced allergic asthmatic mice (H&E staining). Lung sections were obtained from NC mice, sterilized water-treated PC mice, and those treated with different doses of FA. Sections were stained with H&E for the morphological analysis. Tissues were examined by light microscopy (original magnification: ×200). $^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ versus the PC group.

compared to the PC group. Furthermore, in the NC group, cells in the BALF were mostly composed of macrophages (Figure 6(b)). On the other hand, in the PC group, exposure to aerosolized OVA-induced marked increases in cell numbers of eosinophils in the BALF. In contrast, mice administered FA showed significantly reduced increases in eosinophils. Furthermore, a pathological examination of lung tissue sections from the PC group exhibited increased numbers of inflammatory cells around the peribronchiolar region (Figure 6(c)), relative to the NC group. Of note, lungs

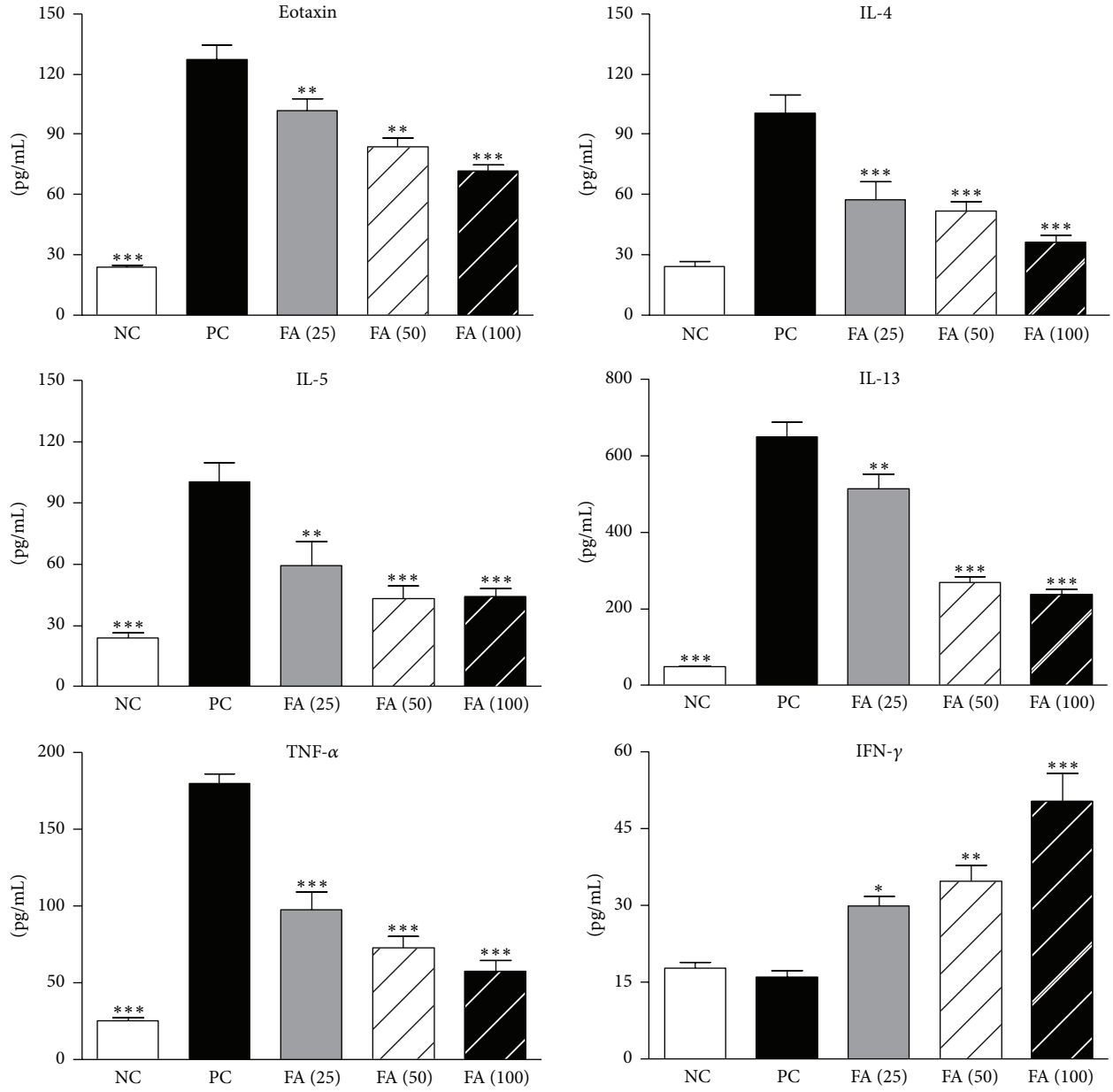


Figure 7: Effect of ferulic acid on levels of chemokines and cytokines in the bronchoalveolar lavage fluid. Immediately after measuring the pulmonary function parameters, each group of mice was sacrificed, and BALF was collected and analyzed for eotaxin, IL-4, IL-5, IL-13, IFN-γ, and TNF-α contents by an ELISA. Results are expressed as the mean ± SEM ($n = 8$ in each group). $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ versus the PC group.

of mice administered FA demonstrated efficient inhibitory effects on peribronchial inflammation.

3.7. The Influence of FA on OVA-Induced Chemokine and Cytokine Secretions in BALF. Eotaxin is the most potent chemokine for recruiting eosinophils. Evaluation of the eotaxin level in BALF showed that administration of FA significantly reduced eotaxin secretion in a dose-dependent manner (Figure 7). This result indicated that FA suppressed eotaxin secretion and subsequently decreased numbers of recruited eosinophils. Furthermore, to determine the possible effect of FA on T-cell responses, we assessed levels of the important Th2 cytokines, IL-4, IL-5, and IL-13, in BALF. The data showed that FA treatment had dose-dependent suppressive effects on the production of Th2 cytokines compared to the PC group. Similarly, the expression level of TNF-α in the BALF also decreased in FA-treated mice. In contrast, we observed significant enhancement of BALF IFN-γ expression in mice treated with FA compared to the PC group.

3.8. Effects of FA on OVA-Induced Cytokine Secretions by Splenocytes. By studying the production of Th1 and Th2 cytokines in cell culture supernatants from the spleen, we determined whether it was possible to affect Th2 immune

responses through inducing IFN-γ-producing T cells. Freshly isolated spleen cells from different groups of mice were cultured with OVA. After 3 days of culture, cell supernatants were collected and analyzed by an ELISA. Results showed that FA administration markedly inhibited IL-4, IL-5, and IL-13 production by splenocytes (Figure 8). Furthermore, the data demonstrated that OVA-sensitized mice treated with FA exhibited an obviously enhanced level of IFN-γ production. We also checked expressions of the proinflammatory cytokines, TNF-α, IL-1β, and IL-6, by splenocytes and found that three dosages of FA treatment significantly reduced production of these cytokines relative to mice in the PC group. Taken together, the above findings suggest that FA treatment might induce protective immunity against established Th2-mediated allergic asthma through induction of IFN-γ production by OVA-specific T cells.

4. Discussion

Asthma affects over 300 million people worldwide and constitutes heavy medical, social, and economic burdens, because its prevalence is continually increasing. Still, in many cases, although corticosteroid and β-agonists can improve asthma symptoms, their long-term use has adverse effects. Therefore, it is imperative to find substances, preferably nonsteroidal in nature and especially those derived from plants used as folk medicines or foods, which have therapeutic effects on allergic asthma and can be taken for a long time with no side effects.

FA is one of the most abundant phenolic acids in plants. It acts as a potent antioxidant which effectively scavenges free radicals and inhibits lipid peroxidation [19]. FA reduced hydrogen peroxide-induced lipid peroxidation in peripheral blood mononuclear cells, and this effect was more evident than that of other polyphenols such as caffeic acid and ellagic acid [20]. FA also increases the activity of enzymes that can counteract free radical-induced damage. Based on this evidence, FA was shown to have a protective role in many disorders, including cardiovascular dysfunctions, cancer, diabetes, and neurodegenerative diseases [21–23]. On the other hand, FA exerts anti-inflammatory properties by affecting the immune system. Hirabayashi et al. found that FA reduced IL-8 production in the influenza virus-infected RAW264.7 macrophage cell line [24]. In the presence of FA, respiratory syncytial virus- (RSV-) infected RAW264.7 cells showed reduced macrophage inflammatory protein- (MIP-) 2 production in a dose-dependent manner [25]. Further, FA inhibited the release of TNF-α from phytohemagglutinin-stimulated splenocytes [26]. These data indicate that FA inhibits inflammation by interfering with immune cells.

In the immune system, DCs are the most potent APCs and are critically involved in initiating primary immune responses and inducing T-cell responses. Thus, elucidating the immunomodulatory effect of FA on DCs could be crucial for understanding the impacts of FA on immune responses. As mature APCs, fully activated mature DCs display high levels of peptide-MHC class II complexes on their surfaces and upregulate surface levels of costimulatory molecules (CD40, CD80, and CD86). These mature DCs are activated through CD40 and produce high levels of IL-12 and TNF-α which drive Th1 cell differentiation [27]. In contrast, differentiation of Th2 cells depends on a low-IL-12 and high-IL-4 environment. IL-4 is not produced by DCs but by other cells, such as mast cells [28]. Similarly, differentiation of Th17 cells is also determined by the cytokine milieu. IL-1β, IL-6, and TGF-β program Th17 cell differentiation [29]. In our study, a FACS analysis of DCs cultured in the presence of FA plus LPS revealed upregulation of MHC class II and CD40 molecules. Further, these FA- and LPS-treated DCs exhibited increased production of IL-12. However, they did not release elevated levels of proinflammatory cytokines, such as TNF-α, IL-1β, or IL-6. Thus, from the above results, we predict that Th1 differentiation may preferably be driven by FA- and LPS-treated DCs. Although it is interesting to discuss which intracellular signaling pathway was involved in modulating gene expression of those cytokines in DCs by FA, further evidence is not available. MAP kinases (MAPKs) consist of three major groups, p38 MAPK, the c-Jun N-terminal kinase 1/2 (JNK1/2), and extracellular signal-regulated kinase 1/2 (ERK1/2), and previous reports indicate that these proteins play a role in regulating cytokine production from APCs [30]. One study reported that LPS instructs DCs to produce IL-12, which depends on the phosphorylation of p38 MAPK and JNK1/2 [31]. Thus, we predict that FA might selectively enhance IL-12 production via amplification of p38 MAPK or JNK1/2 pathway. In addition, FA can act as an antioxidant to reduce proinflammatory cytokine production by scavenging reactive oxygen species (ROSs) [21]. Thus, the inhibition of TNF-α, IL-1β, and IL-6 by FA might be mediated via a mechanism involving the blockage of ROS pathway. In contrast, it is possible that the upregulation of IL-12 production in DCs is through the inhibition of ROS activity. This explanation is supported by one report that H_2O_2, a major component of ROSs, downregulates IL-12 production in murine macrophages in response to stimulation with LPS plus IFN-γ [32]. Further study would be required to clarify this question.

In the current study, we found that FA treatment induced a higher expression level of Dll4 by DCs than those of other Notch ligands. In addition, coculture of $CD4^+$ T cells with LPS-pulsed Dll4-stimulated DCs enhanced the differentiation of Th1 cells characterized by the secretion of increased levels of IFN-γ. Unstimulated DCs express low levels of Notch ligands. Under different conditions, the expression of Notch ligands can be induced towards preferential expression of either Jagged or Delta in response to different stimuli. Amsen et al. demonstrated that expression of Dll4 by DCs was correlated with the ability of LPS to polarize Th1 cells, whereas Jagged1 expression was correlated with the ability of LPS to polarize Th2 cells [5]. The discovery of the contribution of Notch signaling in Th1/Th2 differentiation opens a new pathway for regulating helper T-cell differentiation. Furthermore, Sun et al. showed that ectopic expression of Dll4 by BMDCs promoted Th1-cell differentiation and allowed them to strongly inhibit Th2 development [33]. In addition, inhibition of Th2-type cytokine IL-4 production allows Th cells activated by Dll4 to develop into Th1 cells through a pathway that is IL-12-independent. In summary, our results showed that stimulation via FA can modify the maturation

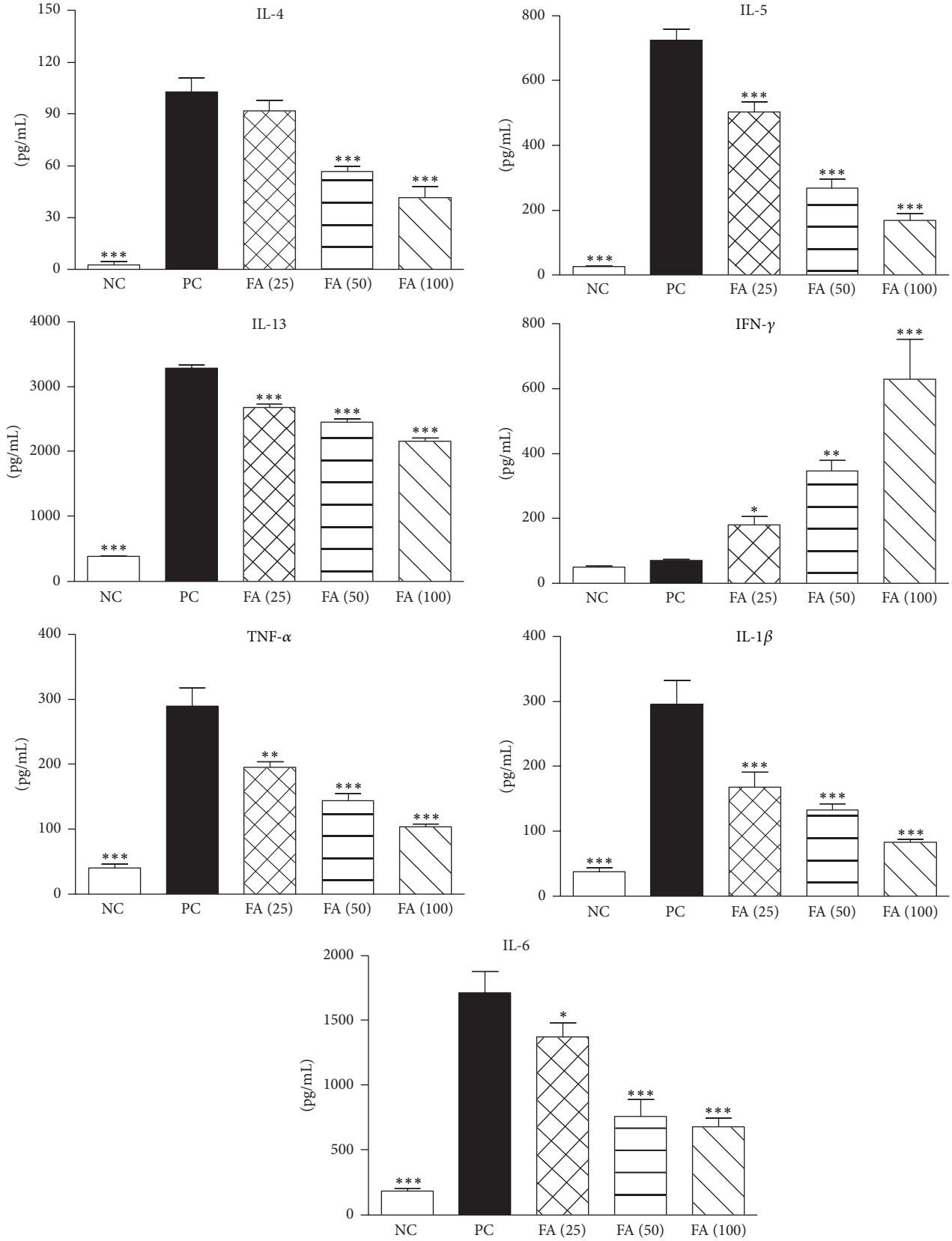


Figure 8: Effect of ferulic acid on the cytokine profile of OVA-restimulated splenocytes. Splenocytes (10^7 cells/mL) from FA-treated and control groups were stimulated with 50 μg/mL OVA in 24-well plates, and culture supernatants were collected after 72 h. Levels of cytokine production of IL-4, IL-5, IL-13, IFN-γ, TNF-α, IL-1β, and IL-6 were analyzed by an ELISA. Results are expressed as the mean ± SEM ($n = 8$ in each group). $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ versus the PC group.

profile and function of DCs, and high expression of Dll4 by these DCs enhanced their ability to activate naïve Th cells and promote Th1 cell development.

In the present study, for the first time, we explored the immunomodulatory effects of FA in a murine asthma model. Our study demonstrated that oral administration of 25 to 50 mg/kg of FA could get significant protective effects against Th2-cell-mediated allergic asthma in mice. FA is of low toxicity with LD_{50} of 2445 mg/kg and 2113 mg/kg body weight in male and female rats, respectively [34]. Although humans may consume as much as 80 to 165 mg FA per meal, low bioavailability after oral administration may limit the clinical use of FA. Another limitation is that many studies on humans were carried out by using foods containing FA rather than the purified FA. Thus, unfortunately, we could not compare with these studies to evaluate the suitable dose of FA applied in humans.

Our results clearly demonstrated that orally administered FA has an antiallergic effect via suppressing OVA-specific IgE production. Serum IgE is one of the most important clinical markers for allergic responses. Such a reduction in serum OVA-specific IgE would likely relieve the allergic syndrome mediated by mast cells [35]. Further, our present results showed that FA treatment significantly induced a reduction in the IL-4 level in mice. IL-4 is a switching factor for IgE production, and it also induces the rolling and adhesion of circulating eosinophils to endothelial cells [36]. Therefore, we speculated that FA may have an antiallergic effect on allergic asthma by suppressing IL-4 secretion which consequently reduces IgE production and eosinophil infiltration into the lungs.

Airway eosinophilic inflammation is one of the characteristics of bronchial allergic asthma. It is believed that eosinophil transmigration into the airways is orchestrated by cytokines, such as IL-4, IL-5, TNF-α, and IL-13, and is coordinated by specific chemokines, such as eotaxin [37, 38]. Specifically, IL-5 is the most important mediator that regulates eosinophilic inflammation through its effects on the proliferation, differentiation, and activation of eosinophils and as a signal for mobilization of eosinophils from the bone marrow into the lungs after an allergen challenge [39, 40]. Additionally, TNF-α and IL-1β are particularly important inflammatory mediators because they play major roles in coordinating mechanisms that command proinflammatory responses. Previous studies reported that levels of TNF-α, IL-1β, and IL-6 released in the BALF of asthmatic patients increased [41]. TNF-α and IL-1β can induce the synthesis of eotaxin in human lung epithelial cells [42]. The results of our study clearly showed that FA attenuated the overproduction of Th2-associated cytokines and downregulated eotaxin expression. Likewise, levels of these proinflammatory cytokines were markedly reduced in FA-treated asthmatic mice. As expected, consistent with results of the histological analysis of lung tissues, FA successfully alleviated lung inflammation and reduced infiltration of total inflammatory cells in peribronchiolar regions. Based on these findings, we speculated that FA inhibits the recruitment of inflammatory cells by reducing levels of Th2-type cytokines, chemokines, and proinflammatory cytokines, providing a possible mechanistic explanation for the observed profound regulatory effects of FA on the development of airway inflammation in this OVA-induced asthma model.

AHR is a major characteristic feature of asthma and a pathophysiologic consequence of the effect of inflammatory processes. In particular, IL-13 can directly cause AHR by affecting airway epithelial cells and airway smooth muscle cells [43, 44]. Herein, we found that BALF and spleen IL-13 levels decreased after oral administration of FA; therefore, it caused attenuation of AHR sensitized by an allergen in mice. IL-10 was shown to be produced by specialized subsets of Treg cells and the regulatory activities of T cells were found to be closely related to their IL-10-producing ability [45, 46]. In this murine asthma model, we detected the expression level of IL-10 in splenocytes. The data showed that FA treatment slightly reduced IL-10 production in splenocytes (Figure S3A). In addition, treatment with FA enhanced but not reduced the OVA-specific T-cell proliferative response (Figure S3B). These results implied that FA treatment did not induce Treg-cell response in this asthmatic animal model.

By administering FA, we demonstrated that FA-treated DCs stimulated T-cell proliferation and enhanced the generation of Th1 cells in an allogeneic MLR. In an animal study, we also showed that treatment of asthmatic mice with FA had inductive effects on Th1 responses, including anti-OVA IgG_{2a} and IFN-γ production. IFN-γ, which is secreted by Th1 cells, promotes the proliferation of Th1 cells and induces IgG_{2a} production by B cells. It inhibits Th2-cell proliferation *in vitro*. Many studies demonstrated that the exogenous delivery of IFN-γ suppresses recruitment of eosinophils and inhibits AHR and bronchial mucus production in asthmatic mice [47–49]. These data support the ability of IFN-γ to block Th2-cell cytokine production and CD4 T-cell recruitment to the lungs. To examine the precise mechanism of suppression, Nakagome et al. delivered IFN-γ to asthmatic mice using an IFN-γ-producing plasmid and reported that IFN-γ attenuated the OVA-induced Th2 immune response by suppressing DC functions, such as antigen presentation, cytokine production, and migration to the lungs [49]. Mitchell et al. used IFN-γ receptor-expressing transgenic mice and showed that IFN-γ acting through the airway epithelium blocked Th2-mediated mucus and inhibited eosinophil generation in the bone marrow [50]. IFN-γ is also considered to play some role in host defense against rhinovirus infection in asthmatic patients. It was speculated that a decrease in its production by peripheral blood mononuclear cells or by lung cells may result in an exacerbation of asthma [51, 52]. These findings suggest that IFN-γ may be beneficial in treating asthma by inhibiting virus-induced exacerbation. Although those studies indicated a novel role for IFN-γ as a broad immune suppressor to modulate immune responses in allergic inflammation, understanding the various mechanisms of IFN-γ regulation in Th2-mediated allergic asthma may define a pathway for new asthma therapies to target in the future.

5. Conclusions

In conclusion, the current study demonstrated the ability of FA to induce maturation of a distinct subset of DCs after

in vitro activation with LPS. In addition, these DCs induced differentiation of IFN-γ-secreting Th1 cells *in vitro*. Furthermore, oral administration of OVA-induced asthma in recipient mice with FA that could lead to differentiation of a specific Th1 population able to downregulate reactivity mediated by IFN-γ production. Therefore, we concluded that the antiallergic effects of FA were due to the modulation of DCs, which promoted the differentiation of naïve T cells into Th1 cells thereby restoring the Th1/Th2 imbalance in a Th2-mediated allergic asthmatic mouse model. Thus, FA might become a very attractive immunomodulatory material that can supplement the human diet. However, further research is necessary to focus on the activation and inhibition mechanisms of FA to clarify its immunomodulatory activities on DCs.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Chen-Chen Lee and Ching-Chiung Wang contributed equally to this work.

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