Baicalin attenuates inflammation in mice with OVA-induced asthma by inhibiting NF-κB and suppressing CCR7/CCL19/CCL21

JIAQI LIU1,2*, YING WEI1,2*, QINGLI LUO1,2, FEI XU1,2, ZHENGXIAO ZHAO1,2, HONGYING ZHANG1,2, LINWEI LU1,2, JING SUN1,2, FENG LIU1,2, XIN DU1,2, MIHUI LI1,2, KAI WEI1,2 and JINGCHENG DONG1,2

1Department of Integrative Medicine, Huashan Hospital, and 2Institute of Integrated Traditional Chinese and Western Medicine, Fudan University, Shanghai 200040, P.R. China

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Abstract. Baicalin, extracted and purified from the Chinese medicinal plant, Scutellaria baicalensis Georgi (Huang qin in Chinese), exhibits potent anti-inflammatory activity against asthma. However, it remains unknown whether baicalin inhibits the activity of CC chemokine receptor 7 (CCR7) and its ligands, which are crucial for the initiation of airway inflammation. In the present study, we investigated the effects of baicalin on CCR7 and its ligands, CCL19 and CCL21, as well as on the nuclear factor-κB (NF-κB) pathway in a mouse model of asthma. A mouse model of acute asthma was established by exposing the mice to ovalbumin (OVA) (by intraperitoneal injection and inhalational challenge). Within 24 h of the final OVA challenge, lung function was detected by direct airway resistance analysis. Lung tissues were examined for pathological changes. Inflammatory cell counts in bronchoalveolar lavage fluid (BALF) were assessed. ELISA was utilized to evaluate the OVA-IgE, CCL19 and CCL21 levels in BALF. The interleukin (IL)-6 and tumor necrosis factor (TNF)-α levels in serum were also detected by ELISA. The protein expression levels of CCR7, as well as that of phosphorylated IκBα (p-IκBα) and phosphorylated p65 (p-p65) were determined by western blot analysis and RT-qPCR was used to determine the CCR7 mRNA levels. Our data demonstrated that the oral administration of baicalin significantly improved pulmonary function and attenuated inflammatory cell infiltration into the lungs. Baicalin also decreased the levels of OVA-IgE, IL-6, TNF-α and CCR7, as well as those of its ligand, CCL19; the levels of NF-κB were also markedly suppressed by baicalin. The CCR7 mRNA level was substantially decreased. Our results thus suggest that baicalin exerts an inhibitory effect on airway inflammation, and this effect may be associated with the inhibition of CCR7 and CCL19/CCL21, which may provide new mechanistic insight into the anti-inflammatory effects of baicalin.

Introduction

Bronchial asthma is a complex inflammatory airway disease mainly characterized by the presence of inflammation, airway hyperresponsiveness (AHR) and airway remodeling. While these are disease-defining characteristics, asthma has recently been recognized as a widely heterogeneous disease [Global Initiative for Asthma (GINA), 2014 update]. To date, it is estimated that 300 million individuals are affected by the disease worldwide and an effective treatment has not yet been found (1). Common allergens such as house dust, animal dander, inhalants, drugs, pollens, and air pollutants contribute to the induction of asthma. Cumulative evidence has indicated that CC chemokine receptor 7 (CCR7) and its ligands, CCL19/CCL21, are involved in these inflammatory responses (2-4); they are also associated with the recruitment and the activation of multiple cell types, such as dendritic cells (DCs), eosinophils (EOS), T cells and B cells (5-8), and also interact with inflammatory cytokines, such as tumor necrosis factor (TNF)-α and interleukin (IL)-6 (9,10).

Chemokines are small (8-14 kDa) chemotactic cytokines that bind to seven transmembrane domain G protein-coupled receptors (GPCR) and are secreted by a great variety of cell types. Chemokines are important mediators involved in the migration and activation of various subsets of leukocytes (11), as well as lymphoid development and lymphocyte homing into and out of secondary and tertiary lymphoid organs.

CCR7 is a member of the GPCR family and is commonly expressed in memory T cells, B cells and mature DCs (12). CCL19 and CCL21 are the only ligands for CCR7. T cells and antigen-presenting DCs establish close physical contacts within the lymph nodes, which jointly contribute to a myriad of events involved in the development of the adaptive immune system.
of adaptive immune functions, including secondary lymphoid organogenesis, and regulatory and memory T cell function (13).

It has been previously described that the CCR7 promoter includes potential binding sites for nuclear factor-κB (NF-κB) (14), which is involved in many inflammatory diseases. CCR7 is a direct target of NF-κB and the induction of CCR7 expression has been shown to be regulated by NF-κB (14), indicating that the modulation of inflammation may be beneficial to the control and treatment of asthma.

Baicalin, as a small molecule flavonoid compound (Fig. 1), which is the major active constituent of Scutellariae radix, has been extensively employed in traditional Chinese medicine (TCM) for the treatment of asthma. It has been demonstrated that baicalin exerts anti-inflammatory effects on experimental colitis (15). In a previous study of ours, we demonstrated the anti-airway remodeling effect of baicalin (16). Moreover, baicalin has been shown to rectify the Th1/Th2 and Treg/Th17 imbalance in asthmatic mice (17). However, the effects and related molecular mechanisms of action of baicalin on CCR7 and its ligands associated with airway inflammation have not been fully elucidated. The present study was designed to examine the effects and the underlying molecular mechanisms of action of baicalin on airway inflammation in a mouse model of ovalbumin (OVA)-induced asthma.

Materials and methods

Animals and reagents. Seven-week-old female BALB/c mice were purchased from SIPPR/BK Laboratory Animal Co., Ltd., (Shanghai, China), and reared under specific pathogen-free conditions following the guidelines and approval of the Animal Care and Use Committee on the Ethics of Animal Experiments of Fudan University, Shanghai, China.

Baicalin (purity ≥98%; chemical structure shown in Fig. 1) was provided by Mast Biotechnology Co., Ltd. (Chengdu, China). OVA (grade V), aluminum hydroxide and methacholine (Mch) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The OVA-specific IgE ELISA kit was obtained from RayBiotech (Norcross, GA, USA). Anti-mouse CCR7 antibody was purchased from Abcam (Cambridge, MA, USA).

Mouse model of OVA-induced asthma and treatment. Asthma was initiated by an intraperitoneal injection of 0.2 ml saline containing 20 μg OVA and 2 mg aluminum hydroxide on days 0, 7, 14 and 21; from day 25 to 31, each mouse was placed into an individual chamber and challenged with 3% OVA nebulization (30 min/day) utilizing an ultrasonic nebulizer model 402AI; Yuyue, Jiangsu, China). 3% OVA nebulization (30 min/day) was initiated by an intraperitoneal injection of 0.2 ml saline containing 20 µg OVA and 2 mg aluminum hydroxide on days 0, 7, 14 and 21; from day 25 to 31, each mouse was placed into an individual chamber and challenged with 3% OVA nebulization (30 min/day) utilizing an ultrasonic nebulizer model 402AI; Yuyue, Jiangsu, China).

The mice were divided into 6 experimental groups as follows (n=12/group): i) the normal control (NC) group; ii) the asthma (A) group; iii) asthmatic mice treated with 10 mg/kg baicalin (B10) group; iv) asthmatic mice treated with 25 mg/kg baicalin (B25) group; v) asthmatic mice treated with 50 mg/kg baicalin (B50) group; and vi) asthmatic mice treated with dexamethasone (DXM) (0.085 mg/kg). The mice in the NC group were sensitized and challenged with an equivalent volume of normal saline. Baicalin and DXM were intragastrically administered from day 24 to 31 prior to the OVA challenge. The following analyses were implemented within 24 h of the final OVA challenge:

Determination of lung function. AHR was detected by an invasive pulmonary facility for small animals (Buxco Electronics, Troy, NY, USA) after the final OVA challenge, as previously described by Pichavant et al (18). Briefly, each mouse was tracheostomized and intubated under anesthesia and the administration by nebulization to gradients of Mch (3.125, 6.25 and 12.5 mg/ml) was carried out to evaluate pulmonary resistance (R1) and pulmonary dynamic compliance (Cdyn). Data were expressed as a percentage change from the baseline value.

Inflammatory cell classification in bronchoalveolar lavage fluid (BALF) and collection of serum. Immediately following the assessment of AHR, and while the mice were still under anesthesia, blood was collected from the left eyeball and stored at 4°C for 2 h and then centrifuged at 5,000 g, 4°C for 30 min. The serum was collected, repackaged and stored at -80°C for use in ELISA. The lung tissues of the mice were then exposed, the right lung was tied and BALF was collected by inserting a tracheal tube and lavaging the left lung 3 times with 0.3 ml aliquots of phosphate-buffered saline (PBS) and centrifuged at 800 x g, 4°C for 10 min. The supernatants were gathered and stored at -80°C for ELISA and the sediments were resuspended in 0.1 ml PBS for determining the cell count using a hemacytometer (Hemavet 950FS; Drew Scientific, Inc., Waterbury, CT, USA).

Lung histopathological assessment. Following the collection of BALF, the mice were euthanized and the lung tissue from the middle lobe of the right lung was detached and fixed in 4% paraformaldehyde, embedded in paraffin and thin slices (4-μm-thick) were cut from blocks and stained with hematoxylin and eosin (H&E). The histopathological changes were determined under an optical microscope (Eclipse 80i; Nikon Corporation, Tokyo, Japan) to assess the inflammatory changes in the lung tissues of the mice.

Histopathological evaluation was performed in a blinded manner on randomized sections. The severity of inflammatory cell infiltration into the lungs was assessed by a 5 point scoring system as previously described by Underwood et al (19).

Detection of IgE, chemokine and cytokine levels in BALF or serum by ELISA. The levels of CCL19, CCL21 and OVA-specific IgE in BALF, as well as the levels of IL-6...
and TNF-α in serum were detected using respective ELISA kits, following the manufacturer's instructions. The assays employed antibodies specific for each cytokine coated on 96-well plates. All reagents, samples and standards were prepared as instructed. Subsequently, 100 µl standard or sample were added to each well followed by incubation for 2.5 h at room temperature. The solution was then discarded and the samples were washed 3-4 times with 1X Wash Solution. This was followed by the addition of 100 µl prepared biotin antibody to each well and incubation for 1 h at room temperature, and further washing with 1X Wash Solution for 3 times. Subsequently, 100 µl prepared streptavidin solution was added following incubation for 45 minutes at room temperature. TMB One-Step Substrate Reagent (100 µl) was then added to each well, followed by incubation for 30 min at room temperature. Finally, 50 µl Stop Solution were added to each well and the absorbance was immediately read at 450 nm.

Reverse transcription-quantitative PCR (RT-qPCR). The expression of CCR7 in the lung tissue was detected by RT-qPCR. Briefly, total RNA was extracted and reverse transcribed into first-strand cDNA. qPCR amplification was then performed according to the manufacturer's instructions using a PCR Amplifier (FTC-3000 qPCR system; Funglyn Biotech, Inc., Toronto, ON, Canada). The cycling conditions were as follows: 1 cycle at 95˚C for 3 min, 40 cycles at 95˚C for 5 sec and 60˚C for 30 sec; and 1 cycle for melting at 94˚C for 90 sec, 60˚C for 3 min and 94˚C for 10 sec. The fold change in the expression of each gene was calculated using the 2^(-ΔΔCt) method, with the housekeeping gene, GAPDH, as an internal control. The final data were presented as the fold change compared to the expression level in the mice in the NC group. The sequences of the primers used are listed in Table I.

Western blot analysis. Lung tissues were collected for the examination of the protein expression of CCR7, as well as that of phosphorylated (p)-IκBα and p-p65 by western blot analysis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (10%) was used to isolate the total protein following extraction. The lung protein samples were electrophoretically transferred onto PVDF membranes. Subsequently, the PVDF membranes were blocked with 5% BSA for 2 h at room temperature followed by incubation with primary and secondary antibodies. The immunoblots were incubated with the following antibodies: primary antibodies overnight at 4˚C with CCR7 (1:2,000 dilution; Abcam), p-IκBα (1:1,000 dilution) and p-p65 (1:1,000 dilution) (both from Cell Signaling Technology, Inc., Danvers, MA, USA); and secondary antibodies at 37˚C for 2 h with 1:2,000 dilution. Finally, the immunoblots were visualized and analyzed using Image Lab software (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Statistical analysis. All analyses were undertaken using SPSS 19.0 statistical software. Data are expressed as the means ± SEM. For comparisons of multiple parameters, ANOVA with the LSD test or Dunnett's test were used to evaluate the statistical significance of the differences between groups. A P-value <0.05 was considered to indicate a statistically significant difference.

Results
Baicalin mitigates AHR in asthmatic mice. The efficacy of baicalin against OVA-induced AHR was examined by means of the airway responsiveness to aerosolized PBS or Mch. As shown in Fig. 2, only inconspicuous alterations in R_L were observed in the normal mice, while the mice with OVA-induced asthma exhibited an obvious increase in R_L and a decrease in Cdyn (Mch of 6.25 and 12.5 mg/ml) compared with the mice in the NC group (P<0.05). Treatment with all concentrations of baicalin led to an obvious increase in Cdyn.
and a reduction in $R_L$ in the asthmatic mice ($P<0.05$) and these effects occurred in a dose-dependent manner.

**Inflammatory cell recruitment in BALF is suppressed by baicalin.** As shown in our results, the OVA-sensitized and challenged mice exhibited a large quantity of inflammatory cells infiltrating the lungs compared to the mice in the NC group ($P<0.01$; Fig. 3). In order to ascertain the effects of baicalin on inflammatory cell counts, we detected general white blood cells (WBCs) and EOS. The WBC and EOS counts in BALF were significantly decreased in the baicalin-treated mice. Moreover, the mice treated with 50 mg/kg baicalin (B50) exhibited a marked reduction in the WBC and EOS counts in BALF ($P<0.01$), and this reduction in inflammatory cell count in the B50 group was greater than that observed in the DXM-treated mice.

**Histological lung inflammation caused by OVA inhalation is reduced in baicalin-treated mice.** In the lung tissues from the mice exposed to OVA, a distinct infiltration of EOS and mucus secretion into the tracheobronchial mucosa and airway lumen was observed as compared with the mice in the NC group ($P<0.05$; Fig. 4). Conversely, the baicalin and DXM-treated mice exhibited less infiltration of EOS into the tracheobronchial mucosa and airway lumen ($P<0.05$).

**Effect of baicalin treatment on IgE, CCL19 and CCL21 levels in OVA-challenged mice.** The results of ELISA indicated...
that the OVA-specific IgE level in BALF was significantly increased by the OVA challenge when compared to the mice in the NC group (P<0.05; Fig. 5A). Gavage of baicalin in each dose group showed an obvious reduction in level of OVA-specific IgE in BALF (P<0.05). Simultaneously, the mice with asthma exhibited a marked increase in the levels of CCL19 and CCL21 (P<0.01; Fig. 5B and C) in BALF. Treatment with 10 mg/kg baicalin (B10) markedly decreased the level of CCL19 and CCL21 (P<0.01), and treatment with baicalin at 25 and 50 mg/kg (B25 and B50) resulted in a marked reduction in the CCL19 level (P<0.05; Fig. 5B). However, treatment with baicalin at 25 and 50 mg/kg did not significantly inhibit the CCL21 level in BALF, whereas DXM did (Fig. 5C).

**Discussion**

Asthma is a highly complex airway inflammatory disorder, the incidence of which is continually increasing. Airway inflammation, AHR and mucus overproduction are the major pathological characteristics of the disease (1). Chemokine receptors with their ligands and NF-κB together with related cytokines are considered to be involved in the pathogenesis of asthma (4,23-25). In this study, the effects of baicalin on inflammation in a mouse model of OVA-induced asthma and on the associated chemokine receptors and their ligands were investigated. Our results revealed that the OVA-exposed mice

![Figure 5. Baicalin reduces ovalbumin (OVA)-specific (A) IgE, (B) CCL19 and (C) CCL21 levels in bronchoalveolar lavage fluid (BALF) of asthmatic mice. OVA-specific IgE and chemokines in BALF were analyzed by ELISA. *P<0.05 vs. normal control (NC) group; **P<0.01 vs. NC group; #P<0.05 vs. asthmatic (A) group; ##P<0.01 vs. A group. B10, B25 and B50 indicates treatment with baicalin at 10, 25 and 50 mg/kg, respectively.](image)

![Figure 6. Baicalin inhibits the expression of inflammatory cytokines in serum of asthmatic mice. (A) IL-6 and (B) TNF-α levels were measured in serum collected from mice in each group. **P<0.01 vs. normal control (NC) group; #P<0.05 vs. asthmatic (A) group; ##P<0.01 vs. A group; ###P<0.001 vs. A group. B10, B25 and B50 indicates treatment with baicalin at 10, 25 and 50 mg/kg, respectively.](image)
exhibited substantial airway inflammatory changes compared to the NC mice. Consistent with previous discoveries (26,27), in this study, AHR was markedly increased in the OVA-exposed asthmatic mice; however, treatment with baicalin led to a significant increase in Cdyn and a reduction in R_L in the asthmatic mice and these effects occurred in a dose-dependent manner, which indicated that baicalin treatment had a protective effect on pulmonary function in the asthmatic mice. Furthermore, the increased amount of inflammatory cells, as well as the OVA-specific IgE level in BALF and the secretion of inflammatory cytokines into the blood of asthmatic mice were markedly reduced by baicalin.

The homeostatic chemokines, CCL19 and CCL21, are the sole ligands for CCR7, and they have a potent chemotactic activity for antigen-presenting mDCs (28) and have been shown to be important in the homing and traffic of naive lymphocytes within lymphoid tissues (29). Studies have demonstrated that the interaction of recruited DCs and T cells at the site of regional...
lymph nodes is promoted by CCL19 and CCL21 (30,31). Mice lacking both CCL21 and CCL19 gene expression exhibit a significantly reduced airway inflammatory response (4). In this study, treatment with various concentrations of baicalin exerted a marked inhibitory effect on the CCL19 level, and treatment with baicalin at 10 mg/kg significantly reduced the CCL21 level in BALF, which may support the anti-inflammatory effect of baicalin in asthma mice.

CCR7 is considered to be involved in the lymph node homing of naive T cells and DCs, and also plays a crucial role in regulating immune cell migration for the balance of immunity against pathogens and the maintenance of central and peripheral immunological tolerance (32,33). It has been shown that CCR7−/− mice exhibit a strongly impaired migration of lung-derived DCs to the bronchial lymph nodes (34). In addition, defective CCR7 expression has been shown to contribute to the reduced efficiency of the interaction between DCs and T cells, as well as to the decreased responsiveness to CCR7 ligands (35). Based on such discoveries, CCR7 has received considerable attention as a potential therapeutic target in allergies (36). In this study, the protein and mRNA expression level of CCR7 were remarkably elevated in asthmatic mice; however, CCR7 protein expression was significantly reduced by treatment with baicalin at 25 and 50 mg/kg and by DXM, and CCR7 mRNA expression was markedly decreased by treatment with baicalin at 25 mg/kg, suggesting that baicalin may be an effective medication for allergic inflammation.

Crucial to the pathogenesis of asthma, the inflammatory cytokines, IL-6 and TNF-α, which have been reported to be implicated in the airway allergic inflammatory responses, regulate many aspects in adaptive immune disorders. IL-6 and TNF-α, which can be expressed by several cell types (24,37), aggregate into the airways and lung tissues when allergy occurs. A previous study demonstrated that IL-6 levels were markedly elevated both in symptomatic and asymptomatic asthma patients (38). A recent study also found that IL-6 trans-signaling increased the expression of airway disease-related genes in airway smooth muscle (25). In addition, the anti-inflammatory effect exerted by targeting TNF-α is currently being widely investigated and studies have shown that the blockade of TNF-α contributes to the suppression of murine allergic airway inflammation (22,39). Various studies have demonstrated that the inhibition of the critical inflammatory cytokines, IL-6 and TNF-α, represent a prominent new approach for the treatment of IL-6- and TNF-α-associated inflammatory diseases (40-43). The findings of the present study demonstrated that compared with the mice in the NC group, the IL-6 and TNF-α levels in serum were significantly decreased by treatment with all concentrations of baicalin, and these effects were more prominent than those observed with DXM treatment. Our data may confirm the hypothesis that baicalin exerts an inhibitory effect on regulatory Th17 cells in asthmatic mice.

NF-κB is a master regulatory transcription factor that is crucial to the expression of inflammatory cytokines, chemokine receptors and the counterpart ligands in inflammation-related diseases (44). The inhibitor of IκB kinase (IKKs), p65/p50 complex and IκB are key cytokines in the NF-κB pathway; however, among the p65/p50 complex, only p65 has transcriptional activity (45). It has been demonstrated that the interaction between CCR7 and its ligand, CCL19, activate p-IκB and cause NF-κB to translocate to the nucleus, increasing the DNA-binding capacity of NF-κB (46). In addition, it has been demonstrated that the stimulation of DCs with CCL19 or CCL21 induces the activation of NF-κB (47). Therefore, CCR7-mediated NF-κB activation may play a key role in the pathophysiological process of asthma (Fig. 8), and the suppression of NF-κB activation and the interaction with CCR7 and its ligands are associated with reducing airway inflammation. Accordingly, we investigated whether baicalin treatment could interfere with CCR7 and the NF-κB pathway. The data from western blot analysis revealed that the expression of p-IκBα and p-p65 protein were markedly elevated in the lung tissue of OVA-exposed mice as compared to the NC mice and were markedly inhibited by treatment with baicalin at the dose of 25 mg/kg. Collectively, these data suggest the potential of baicalin to inhibit the functions of CCR7 and its ligands via NF-κB, thus attenuating inflammation, and the development and progression of asthma.

In conclusion, in the present study, we demonstrated that baicalin improves pulmonary function and attenuates airway inflammation. Our data provide novel mechanistic insight into the anti-inflammatory effects of baicalin. Importantly, the levels of IgE and CCR7, and those of its ligand, CCL19, were prominently suppressed by treatment with baicalin. Furthermore, baicalin exerts an inhibitory effect on the NF-κB pathway. These discoveries may demonstrate the potential of baicalin as an agent for the management of allergic asthma.

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References


