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Modulation of the airway smooth muscle phenotype in a murine asthma model and effects of nuclear factor- κ B inhibition

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ABSTRACT

Objective: Phenotype modulation of airway smooth muscle (ASM) is a unique characteristic of asthma and is considered to regulate airway remodeling, airway hyperresponsiveness (AHR) and inflammation. The nuclear factor- κ B (NF- κ B) signaling pathway plays a crucial role in phenotypic modulation. Thus, models of acute and chronic asthma were established and pyrrolidine dithiocarbamate (PDTC), an NF- κ B inhibitor was delivered by intraperitoneal injection. **Methods:** The Penh value was measured using the BUXCO WBP system. Lung tissues were subjected to histologic analysis. Phenotypic markers of ASM and COL1A1 mRNA levels were measured by RT-PCR. Expression levels of phosphorylated p65 (pP65) and α -SMA were detected by Western blot. Serum cytokine levels were quantified by RayBiotech ELISA array. **Results:** PDTC intervention decreased the Penh values in both the acute and chronic models. The ASM area and the airway collagen area were decreased in the PDTC intervention group. A decrease in phenotypic markers were detected in both the acute and chronic models in time-dependent manner, and PDTC intervention partially reversed the phenotypic modulation. The effect of PDTC intervention on systemic inflammation was also verified. **Conclusion:** These results revealed the existence of a dynamic ASM phenotype modulation procedure in asthma development and that targeting NF- κ B by PDTC was effective to mitigate ASM phenotype modulation and major asthmatic pathological features.

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Introduction

Asthma is one of the most prevalent chronic respiratory diseases worldwide and affecting more than 300 million people currently. The number of cases is estimated to increase to 400 million by the year 2025 [1]. Conventionally, airway inflammation is believed to be the central pathogenesis of asthma. Airway hyperresponsiveness [AHR] and airway remodeling deteriorate as continuous inflammation stimulation in airway structures [2]. In recent years, accumulating investigations revealed that the non-immune components of airways are independent participants rather than a concomitant consequence of chronic inflammation in the development of asthma [3]. In this context, airway smooth muscle (ASM) might play a pivotal role as it is the main component of airways and exhibits great

potential in the repair-injury response of airway tissue and immune regulation [4].

The diverse functional and structural alterations of ASM were defined as phenotype modulation. Previous *in vitro* cell studies revealed that the exposure of ASM to a series of mitogens lead to reversible changes from contractile phenotype to synthetic status with an increased proliferation rate, excessive synthetic capabilities and reduced contractile smooth muscle marker protein [5]. These abnormal ASM functional modulations are closely related to disease hallmarks of asthma. However, few studies have assessed whether *in vitro* phenotype modulation recapitulates the actual condition *in vivo*. In addition, the underlying molecular mechanisms of ASM modulation have been investigated extensively in *in vitro* experiments but have not been fully assessed *in vivo*.

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Recently, the transcription factor nuclear factor- κ B (NF- κ B) has drawn great attention as a therapeutic target of chronic lung disease due to its role in mediating inflammation-related signaling events [6]. Moreover, several lines of evidence indicate that NF- κ B mediates VSMC proliferation and synthesis in vascular remodeling [7,8]. It is reported that the NF- κ B signaling pathway plays a crucial role in ASMC phenotypic modulation as indicated by restored contractile markers after NF- κ B intervention [9]. Based on the above issues, the current study aimed to explore the development of ASM phenotype modulation and the effect of in vivo inhibition of NF- κ B using a validated murine model of asthma. In addition, we study the NF- κ B inhibition on airway remodeling, AHR and chronic inflammation, thus providing a theoretical and experimental basis for the search for new asthma treatments.

Materials and methods

Mice

Female BALB/c mice (6 weeks old) were purchased from Vital River Laboratory Animal Technology Co. (Beijing, China) and maintained under environmentally controlled, specific pathogen-free conditions throughout the duration of the studies. All procedures were performed in compliance with the guidelines of Guangdong province, and the protocol was approved by the IRB committee of Shenzhen Institutes of Advanced Technology, Chinese Academy of Sciences.

Sensitization and airway challenge

On days 0 and 7, mice were sensitized by intraperitoneal injections of 20 ovalbumin (OVA, chicken egg albumin, grade V, Sigma, St. Louis, MO, USA) + 50 μ l of Imject® Alum (Thermo Fisher Scientific, Boston, MA, USA) followed by daily 30-min 5% aerosol grade II OVA (Sigma, St. Louis, MO, USA) challenges for 5 consecutive days (acute model) or thrice weekly for 6 weeks (chronic model). Control groups were administered saline in the same manner. Mice were treated with pyrrolidine dithiocarbamate (PDTC, Sigma, St. Louis, MO, USA) or vehicle by i.p. injection 2 h before challenge with aerosolized OVA. The dosage (5 mg/kg) was determined based on a preliminary study and had the least side effects. Mice were analyzed 24 h after the last antigen challenge inhalation, and then the serum and lung tissues were harvested for further studies.

Table 1. The primer sequences of target genes for Q-PCR.

Gene name	Primer sequences (5'-3')
α -SMA	Forward primer CTGGAGAAGAGCTACGAACTG Reverse primer CTGATCCACATCTGCTGGAAG
Calponin	Forward primer TCTGCACATTTAACCAGAGGTC Reverse primer GCCAGCTTGTCTTTACTTCAGC
SM-MHC	Forward primer GTGTGGTGGTCAACCCCTAC Reverse primer GATGTGAGGCGGCATCTCAT
COL1A1	Forward primer GCGGTAACGATGGTGTCTTT Reverse primer CTTACCCCTTAGACCAAC
β -Actin	Forward primer GAGACCTTCAACACCCAGC Reverse primer ATGTCACGCACGATTTCCT

Airway responsiveness to MCh

Airway responsiveness was measured 24 h after the last aerosol exposure by recording respiratory pressure curves using barometric unrestrained whole-body plethysmography (Buxco; EMKA Technologies, Paris, France) in response to inhaled methacholine (MCh, Sigma, St. Louis, MO, USA) in conscious unrestrained mice. Airway responsiveness was expressed as the enhanced pause (Penh) as previously described in detail (Hamelmann et al., 1997). Briefly, mice were placed in a whole-body chamber, and basal readings were obtained and averaged for a 3-min period. Subsequently, increasing doses of methacholine (0–100 mg/ml) were aerosolized for 3 min, and readings were obtained and averaged for 3 min after each nebulization.

Histology

Right upper lobes and right middle lobes were fixed in 4% paraformaldehyde for 24 h. Tissues were then paraffin-embedded and cut into 5-mm sections. Sections were stained with hematoxylin and eosin to assess inflammatory infiltration and with Masson trichrome to examine airway fibrosis. An antibody against calponin (1:200, Abcam, Cambridge, UK) was used to visualize smooth muscle cells (SMCs). Three inconsecutive sections were observed, and 5 to 7 high-resolution digital images were obtained from areas containing medium bronchi (area internal to the basement membrane is 50 000–175 000 mm²). The ASM area was quantified using ImageJ (National Institutes of Health, Bethesda, MD, USA). Data were normalized to the Pbm² to correct for airway size.

Real-Time polymerase chain reaction (qPCR)

Lung tissues were gently ground with a plunger of a syringe and a stainless-steel filter mesh; intrapulmonary airways were then isolated carefully under a stereoscopic microscope using microsurgical instruments. Total RNA was isolated according to the

protocol for TRIzol (Invitrogen, Carlsbad, USA) and then reverse transcribed into cDNA using the ReverTra Ace qPCR RT Master Mix with gDNA Remover (Toyobo, Osaka, Japan). The primer sequences are listed in Table 1. Quantitative real-time PCR was performed with the use of a QuantiFast SYBR Green PCR Kit (Invitrogen, Carlsbad, USA) on a Roche LightCycler 480II system. Target gene expression was normalized to β -actin using the $2^{-\Delta\Delta CT}$ method.

Western blot

Isolated intrapulmonary airways were disrupted using a homogenizer and lysed in buffer. A BCA assay was performed to determine the protein content. Protein extracts and broad-range molecular weight markers were then separated by 8 to 12% (weight/volume (w/v)) sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, 10%) and transferred to PVDF membranes. After blocking, the membranes were incubated overnight at 4°C with monoclonal anti-mouse α -SMA (1:1000, Abcam, Cambridge, UK) and anti-mouse p-NF- κ B p65 (1:1000, Danvers, USA) antibodies in TBST containing 5% nonfat powder milk. The next day, the membranes were washed and incubated with the corresponding secondary antibody conjugated to HRP (1:2000, Abcam, USA) for 90 min. Antibodies against β -actin (1:10,000, Sigma, St. Louis, MO, USA) were used as the protein loading control. Blots were visualized with enzyme-linked chemiluminescence (ECL Plus, Beyotime, Shanghai, China) followed by densitometry.

Protein microarray of serum inflammatory cytokines

The cytokine levels in the serum were measured with Mouse Interleukin Array Q1 (RayBiotech, Norcross, USA) according to the manufacturer's instructions. The fluorescently labeled array was visualized using InnoScan 300 (innopsys, Carbonne, France). Data were extracted by GenePix Pro 5.1 software and analyzed by RayBiotech Q-Analyzer Software for QAM-INT-1.

Data analysis

All data are expressed as the mean $\pm$ SEM. Statistical analyses (two-sample t test, one-way ANOVA and two-way ANOVA with Tukey's post-tests) were performed with GraphPad Prism 5 (GraphPad, La Jolla,

CA, USA). $p < 0.05$ was used to indicate a statistically significant difference.

Results

Inhibition of NF- κ B by PDTC treatment reduces the development of airway hyperresponsiveness and prevents airway smooth muscle phenotype modulation in both chronic and acute asthma

To investigate the effects of PDTC on airway function, airway hyperresponsiveness was measured by whole-body plethysmography with increasing doses of methacholine. As shown in Figure 1(A), the mean absolute Penh value was augmented in the OVA + vehicle mice compared with that of the respective naive controls, whereas PDTC treatment significantly reduced the mean absolute Penh value compared with that of the OVA + vehicle mice in both acute and chronic models.

Three genes encoding the contractile proteins of ASMC were studied by real-time PCR: α -smooth muscle actin, calponin and SM-MHC. α -Smooth muscle actin, calponin and SM-MHC mRNAs were dramatically decreased in OVA + vehicle mice compared with those in naive controls in the chronic model (Figure 1(B)). In the acute model, only one out of three genes (calponin) was down-regulated with statistical significance in OVA + vehicle mice (Figure 1(C)). PDTC treatment partially reversed the down-regulation of contractile gene expression in both the acute and chronic models. We also assessed COL1A1 mRNA expression in the intrapulmonary airways. Although OVA + vehicle mice demonstrated increased expression of COL1A1 that was significantly decreased by PDTC treatment in the acute model, there were no significant differences between the groups in the chronic model.

Effect of PDTC on protein levels of phenotype markers in acute and chronic models of allergic asthma

We further determined the relationship between NF- κ B pathway activation and ASMC phenotype switching by Western blot in the chronic models. As shown in Figure 2, the levels of phospho-p65, which indicate successful inhibition of classical NF- κ B activity, were reduced by PDTC treatment. In addition, relatively high levels of α -smooth muscle actin were noted in the OVA + PDTC group compared with those in the OVA + vehicle group.

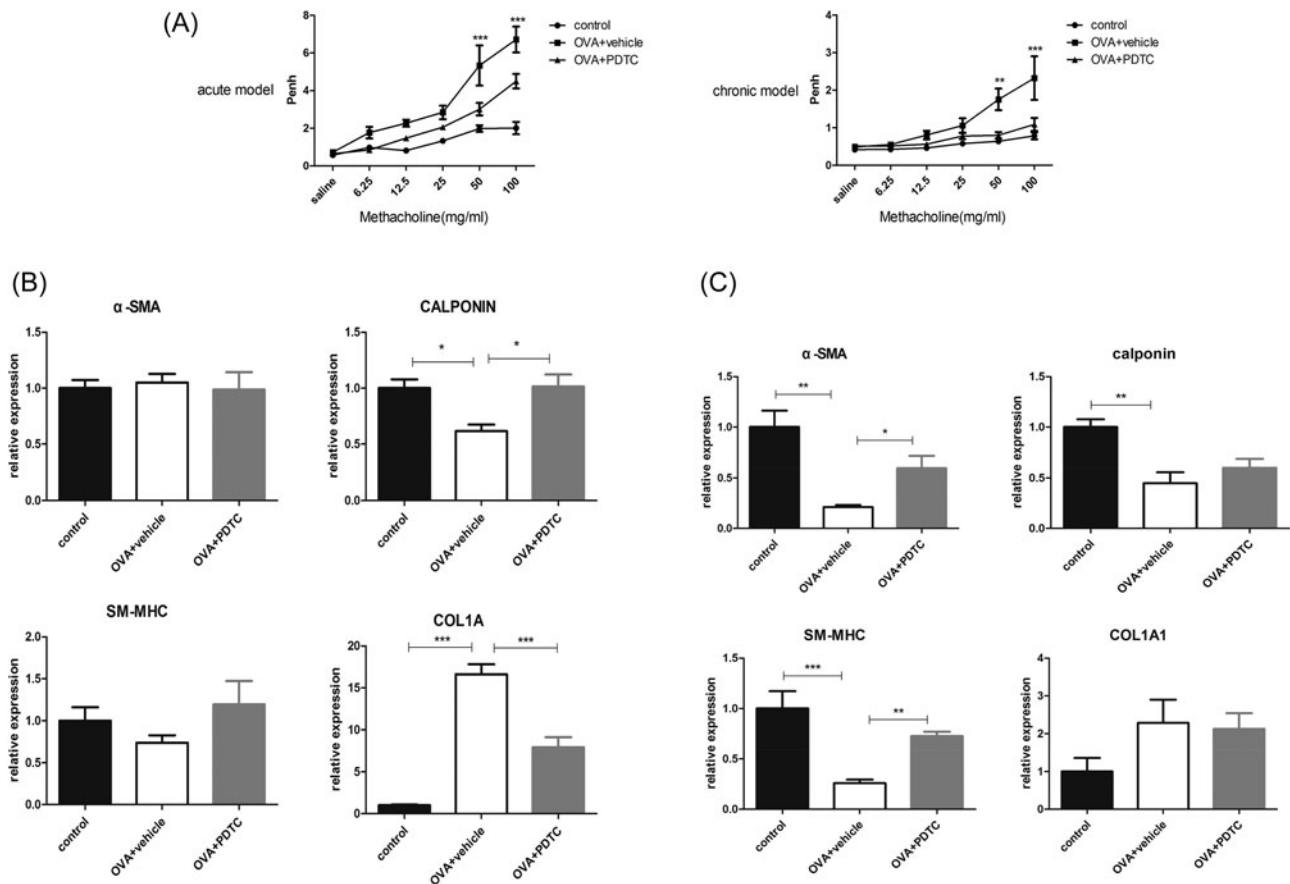


Figure 1. Penh changes to Mch and effect of PDTC on mRNA expression of phenotype markers in acute and chronic models of allergic asthma. Penh value changes in acute and chronic models (A) and mRNA expression of phenotype markers in acute (B) and chronic models (C). Values are expressed as the means $\pm$ SEM. ** $p < 0.01$ and *** $p < 0.001$, $n = 6$.

Effect of NF- κ B inhibition by PDTC treatment on allergen-induced airway remodeling

In the chronic model, dense collagen deposition was observed throughout the lung interstitium surrounding the airways and blood vessels compared with that in the naive control mice. PDTC treatment markedly reduced collagen deposition in the lung interstitium of OVA-treated mice, including perivascular collagen deposition. Morphometric analysis showed that the collagen deposition area was significantly increased in OVA + vehicle mice compared with that in the control mice. Significantly, PDTC treatment reduced the collagen deposition area in OVA + PDTC mice compared with that in the OVA + vehicle group (Figure 3(A)) and was not significantly different from that of the saline-treated controls. No difference in collagen deposition was noted between the groups in the acute model (data not shown).

The effect of NF- κ B inhibition on the OVA-induced increase in ASM was investigated. We immunostained lung sections for calponin and subtracted this signal from the remainder of the lung tissue using

image analysis software (Figure 3(B)). The ASM mass was expressed as the area of tissue normalized for airway size using the basement membrane perimeter (BMP). In the chronic model, a significant increase was evident in the ASM mass of small airways in the OVA + vehicle mice versus naive control mice. This increase in ASM mass was significantly suppressed by treatment with PDTC. In contrast, in the acute model, no significant differences were observed in ASM mass between each group (data not shown).

Effect of NF- κ B inhibition by PDTC treatment on allergen-induced airway inflammation

Hematoxylin and eosin staining revealed extensive inflammatory cell infiltrate around the pulmonary blood vessels and airways in the OVA + vehicle mice compared with naive control animals, which resolved substantially in the OVA + PDTC group (Figure 3(C and D)). To determine the effect of PDTC on cytokine production in asthma models, the levels of serum cytokine expression were profiled using a Raybiotech quantitative multiplex Elisa array (Figure 4). In the

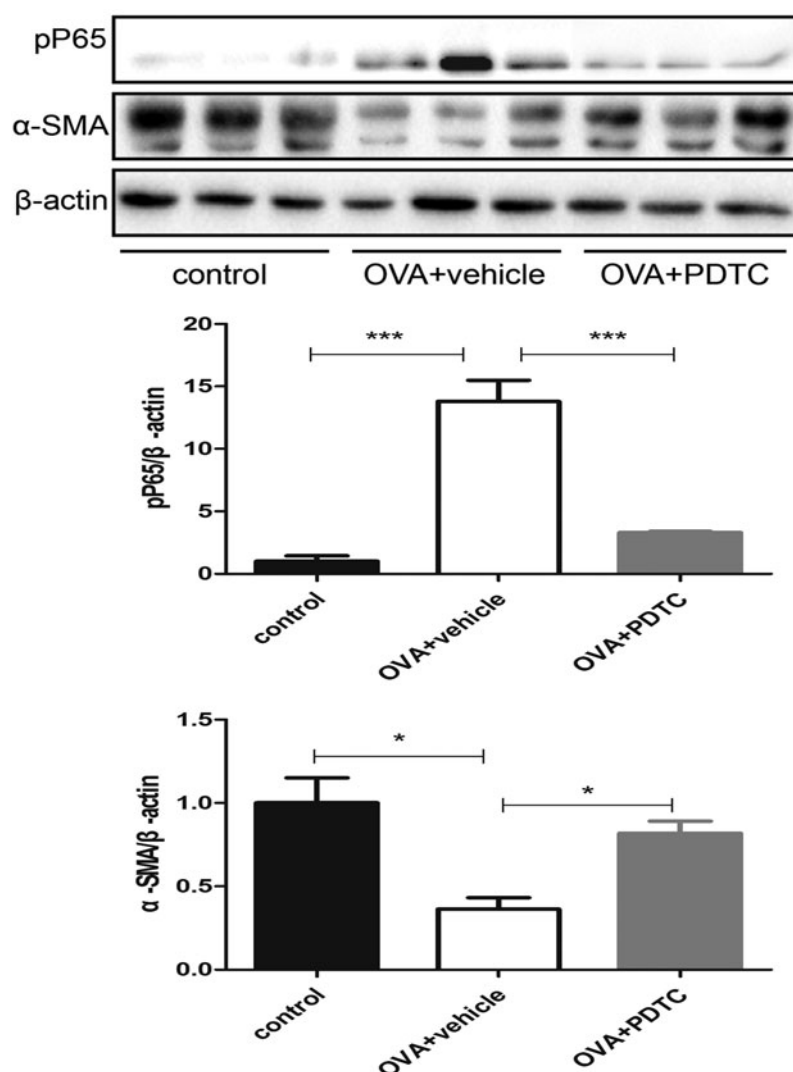


Figure 2. Effect of PDTC on protein levels of phenotype markers in acute and chronic models of allergic asthma. PDTC treatment blocked the activation of p65 and the loss of the contractile mass in chronic models of allergic asthma. Values are expressed as the means $\pm$ SEM. * $p < 0.05$ and *** $p < 0.001$, $n = 6$.

acute model, G-CSF, IL-6 and IL-17 were significantly increased in OVA + vehicle mice compared with naive controls, whereas PDTC treatment significantly reduced G-CSF and IL-6 levels but not IL-17 levels. In the chronic model, only IL-17 and TNF- α were significantly increased in OVA + vehicle mice, and TNF- α was reduced in OVA + PDTC mice. Although IL-6 was increased in the chronic model and was reduced by PDTC treatment, statistical analysis indicated no significance.

Discussion

In this study, we identified a temporal sequence that was involved with ASMC phenotype modulation. Specifically, the contractile protein was reduced in a time-dependent manner during asthma progression.

By morphometric analyses, we demonstrated a significant increase in ASM mass and collagen deposition in a chronic but not acute murine model of asthma. However, excessive proliferation and collagen synthesis was noted only in the acute model. Inhibition of NF- κ B signaling by systemic administration of PDTC partially reversed ASMC phenotype switching and the histopathological features relevant to ASMC dysfunction mentioned above. In addition, airway hyper-responsiveness and cytokine production were also attenuated by NF- κ B inhibition in the acute and chronic models, suggesting that NF- κ B might act as a junction point in the regulation of the ASMC phenotype and airway inflammation.

ASMC phenotype modulation refers to the ability to switch from a contractile phenotype to a proliferative/synthetic phenotype that is accompanied by a series of

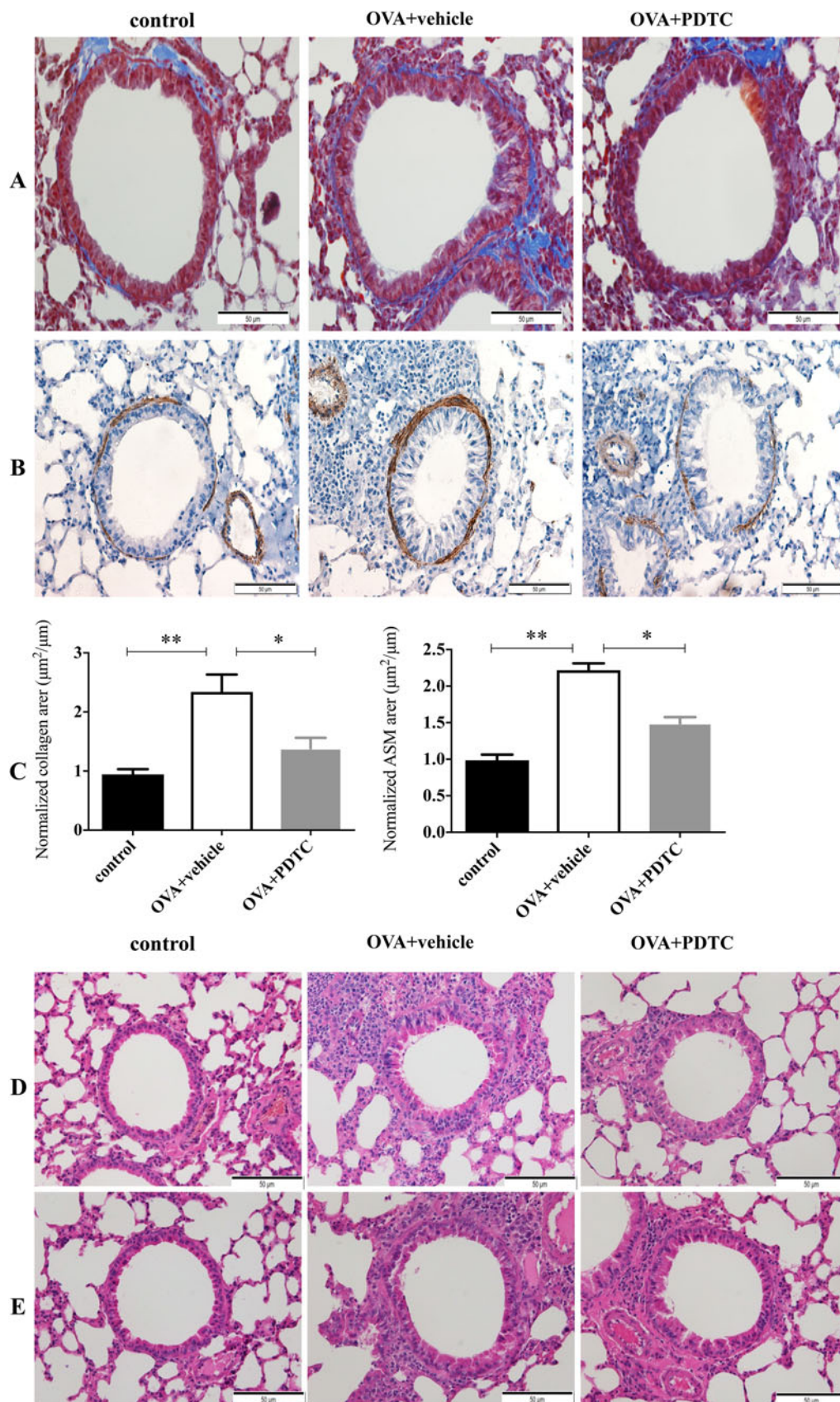


Figure 3. Effect of PDTC on collagen deposition, airway smooth muscle mass accumulation and inflammatory cell infiltration in airways models of asthma. Representative pictures of Masson trichrome (A) and smooth muscle-specific IHC (B) staining. (C) The analysis of Masson trichrome and smooth muscle-specific IHC. Values are expressed as the means $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$, $n = 6$. Hematoxylin and eosin-stained sections of lungs from acute (D) and chronic models (E).

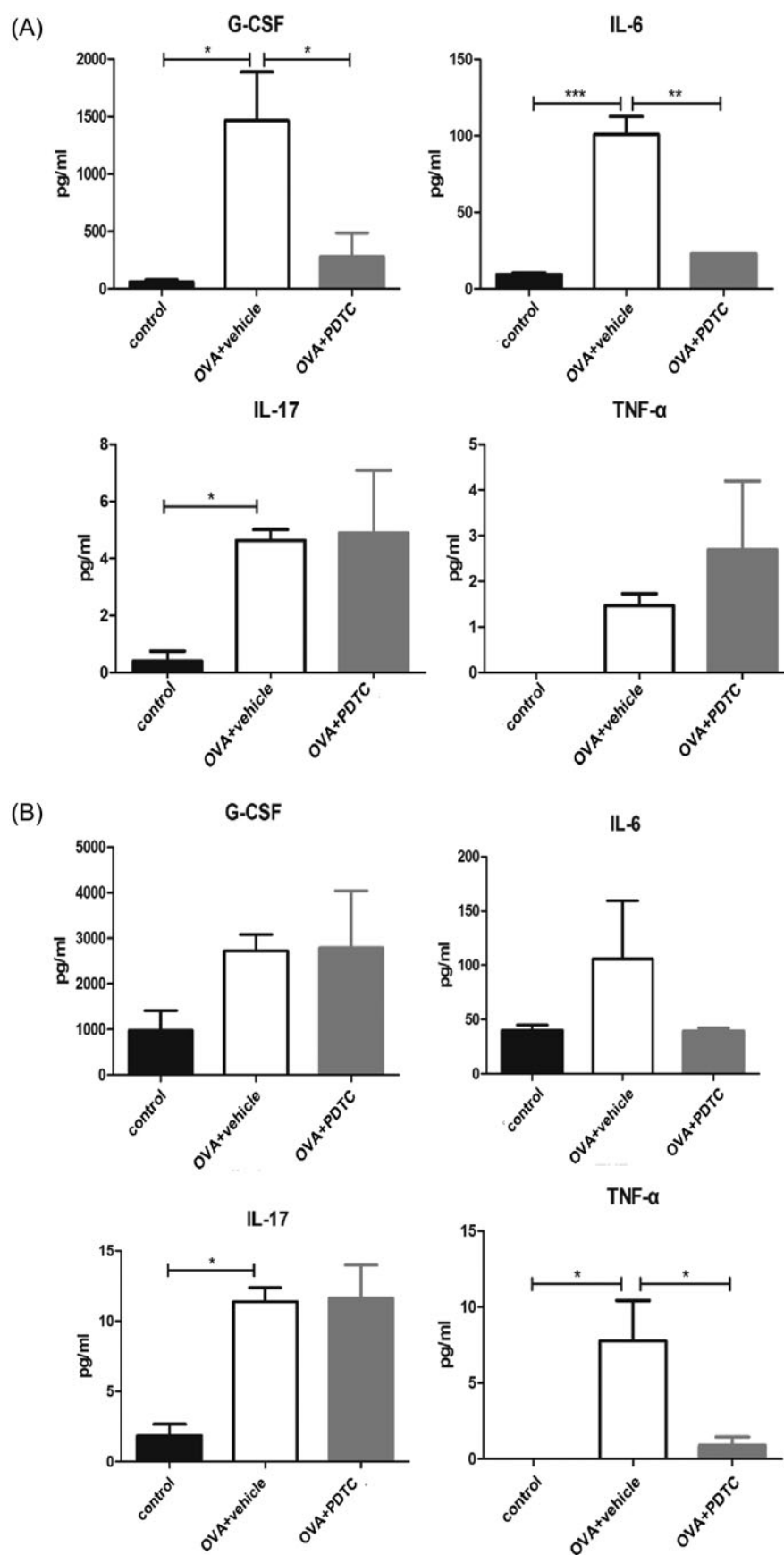


Figure 4. Effect of PDTC on levels of inflammatory cytokines in acute and chronic models of allergic asthma. A quantitative cytokine array was used to measure the protein levels of inflammation markers in acute model (A) and chronic model (B). Values are expressed as the means $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$, $n = 3$.

functional changes. Despite intensive investigations *in vitro*, the *in vivo* existence and consequences of ASMC phenotype modulation are far from understood. Previously, Hirst and his colleagues reported reduced expression of the contractile mass and altered composition of the contractile apparatus [10] in a rat model of asthma. In contrast, other studies failed to detect contractile mass reduction [11], making this issue complicated and confusing. However, these inconsistent results are not directly comparable because the latter study separately assessed large airways, whereas different regions of the tracheobronchial tree were likely to exhibit diverse responses to allergen exposure. More recently, Batt and his colleagues demonstrated the temporality of the ASMC response in a murine model of asthma with an initially proliferative and antiapoptotic reaction, the subsequent development of hypertrophy and an increased muscle mass [12]. This phenomenon might represent the functional modulation of ASM. However, the work did not assess the change in ASMC phenotypic markers during chronic OVA challenge.

To better understand the dynamic relationship between ASM phenotype modulation and its functional alterations, we simultaneously examined phenotypic markers and ASM-related asthmatic features in the acute and chronic murine asthma models. Decreased α -smooth muscle actin, calponin and SM-MHC mRNA levels were observed after 6 weeks of repeated OVA challenge, and only calponin was reduced by 5 consecutive days of OVA challenge. Another study reported the occurrence of ASM phenotype modulation in a rat asthma model at a point (24 day) between our acute and chronic models [13]. In combination with the current results, we reasoned that the *in vivo* progression of ASM phenotype modulation is a dynamic procedure wherein the contractile protein is reduced in the acute phase and later decreases to a much greater extent in the chronic phase. The abnormal phenotype transformation (from contractile phenotype to synthetic phenotype) of airway smooth muscle is a key link in the airway remodeling of asthma. When the mRNA and protein level of contractile markers decreased in asthma, suggesting that synthetic phenotype markers expression elevated and resulted the thicken of the ASM layers.

Paralleling these findings, histopathological changes of ASM and collagen deposition also occur in sequential phases. Prominent proliferation and increased COL1A1 expression was present in the acute but not in the chronic model and was not confined to ASM. Previous studies demonstrated that the airway inflammatory reaction was dominant in the early phase of the allergic asthma model, and then tolerance to the

allergen developed during chronic exposure [14,15]. The waning of the proliferative and synthetic responses can be explained by the absence of an inflammatory stimulus. Nevertheless, the contractile and structural abnormalities of ASM sustained in the chronic stage indicate that the regulatory pathways independent of airway inflammation are involved in ASMC dysfunction.

The mechanisms of ASM phenotype modulation have not been fully elucidated. However, an intracellular signaling network imbalance due to intrinsic or acquired abnormalities seems to play a crucial role [16]. In our previous work, we demonstrated that p65, a subunit of NF- κ B, was overactivated in ASM isolated from a mouse model of OVA-induced allergic asthma. Inhibition of NF- κ B prevents asthmatic ASM from the loss of the contractile mass and simultaneously inhibits proliferation and promotes apoptosis [9]. The production of various cytokines by ASM through an NF- κ B-dependent mechanism was also suggested by other investigators [17]. More recently, a study on cardiovascular disease revealed that NF- κ B cell autonomous activation mediates vascular smooth muscle cell phenotypic modulation by repressing myocardin, a strong transcription factor that regulates the expression of the VSMC contractile genes by physically interacting with it or binding to its promoter region with Klf4 [18]. Taken together, we hypothesized that the NF- κ B signal pathway may act as a junction point that integrates ASM phenotype markers and cellular function alteration.

Via systemic administration of PDTC, the current study demonstrated that inhibition of the NF- κ B pathway partially reversed AHR, ASM phenotype modulation and the relevant histopathological changes in murine models of asthma, which is consistent with other investigations [19]. Given that the methods of NF- κ B intervention in most of the studies were not specific for ASM, it is possible that the benefit was not a result of targeting ASM. In fact, a recent study reported that activation of classical and alternative NF- κ B pathways occurs within the airway epithelium and may coordinately contribute to allergic inflammation, AHR and fibrotic airway remodeling [20]. However, ASMC is a major contributor to the pathophysiology of asthma. AHR can be dissociated from airway inflammation and partially results from ASM phenotype modulation [21]. Despite a reduction in the contractile mass, the force generation of ASM is increased in the chronic asthmatic model and may be explained by intracellular calcium dysregulation. NF- κ B is involved in Ca(2+) homeostasis through the regulation of Ca(2+) channels or PKC-

potentiated inhibitory protein of 17 kDa (CPI-17) transcription [22] and could be an underlying mechanism that inhibits AHR by NF- κ B targeting treatment.

Previous studies demonstrated that the NF- κ B pathway, which plays a key role in a broad spectrum of inflammatory networks, was persistently activated and thereby resulted in aberrant sustained expression of inflammatory genes in asthmatic lung tissue [23]. Our studies and the work of other laboratories also revealed that asthma is not only an inflammatory response limited to the airways but also contains systemic inflammation components [24,25]. Treatment with PDTC reduced the levels of IL-6 and G-CSF in serum and may have a certain impact on TNF- α production. However, we noticed that PDTC treatment had no effect on IL-17 expression. Some research groups report that NF- κ B activation positively regulates Th17 cell differentiation [26,27], whereas others showed that NF- κ B activation does not affect or negatively regulate Th17 cell differentiation [28,29]. Our results may provide additional information that NF- κ B activation is not responsible for Th17 cell differentiation or secretion of IL-17 in asthmatic inflammation.

However, there are some limitations in the current study. Our research focuses on the effect of the intraperitoneal injection of PDTC upon the asthma. The cause of asthma is complicated and it is hard to get a good result by merely targeting a single mechanism. Intracardial injection is more practical to drug delivery in clinic, which could influence the airway inflammation, airway remodeling, airway hyper reactivity, etc. However, this kind of administration method cannot verify the PDTC specific inhibits the activation of NF- κ B in smooth muscle skin cells clearly. On the other hand, the toxicity of PDTC also limits its application.

In summary, we demonstrated that the *in vivo* progression of ASM phenotype modulation that accompanies cell function alteration is a dynamic procedure. In addition, the contractile protein levels were reduced in the acute phase and were later decreased to a much greater extent in the chronic phase. Next, NF- κ B pathway interventions reversed the airway smooth muscle cell phenotype modulation to some extent. Finally, NF- κ B signaling pathway intervention may alleviate asthmatic airway remodeling, airway hyperresponsiveness and chronic inflammation to some extent and therefore can serve as an intervention target for asthma therapy.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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