

## Research Article

# NLRP3 regulates macrophage M2 polarization through up-regulation of IL-4 in asthma

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Activation of nucleotide-binding oligomerization domain-like receptor protein 3 (NLRP3) inflammasome received substantial attention recently in inflammatory diseases. Macrophages contribute to allergic inflammation in asthma. The present study was aimed to investigate the effect of NLRP3 inflammasome on the polarization of macrophages. We utilized human primary monocytes and monocyte-derived macrophages to study the expression of NLRP3 inflammasome components (NLRP3, apoptosis-associated speck-like protein, and caspase-1) and its downstream cytokine interleukin-1 $\beta$  (IL-1 $\beta$ ). By gain- or loss-of-function assays, we next explored the effects of NLRP3 inflammasome on M1/M2 polarization and secretion of IL-4, interferon- $\gamma$ , tumor necrosis factor- $\alpha$ , and IL-1 $\beta$ . The results showed increased numbers of M2 cells in asthma. And NLRP3 inflammasome was activated and involved in the inflammation of asthma. Furthermore, silence of NLRP3 down-regulated IL-4 secretion and up-regulated M1/M2. In contrast, overexpression of NLRP3 increased IL-4 and decreased M1/M2. As expected, IL-4 was involved in NLRP3-mediated down-regulation of M1/M2 ratio. Moreover, NLRP3 interacted with IRF4 and was required for optimal IRF4-dependent IL-4 transcription. Subsequently, deficiency of NLRP3 in ovalbumin-induced allergic asthmatic mice impaired lung inflammation and up-regulated M1/M2, and diminished IL-4 in bronchoalveolar lavage fluid. Collectively, we demonstrated here that activation of NLRP3 was engaged in the promotion of asthma. NLRP3, but not the inflammasome adaptor ASC or caspase-1, promoted the polarization of M2 macrophages through up-regulating the expression of IL-4, thereby contributing to its regulation of asthma.

## Introduction

Asthma is a heterogeneous inflammatory disorder of the lungs characterized by chronic airway inflammation and bronchial hyper-responsiveness [1]. Asthma is a major public health problem and has increased dramatically in prevalence over the past decades in western countries [2]. So far, the pathogenesis of asthma has still not well been documented. However, there are accumulating evidences, suggesting that it is closely related to the immune system [3,4].

Macrophages are critical mediators of innate immune responses [5]. According to different functions after activation, macrophages can be divided into classically activated macrophages (M1) and alternatively activated macrophages (M2) [6]. In response to cytokines or microbial products present in the microenvironment, macrophages can change their phenotype and be polarized to M1 or M2 macrophages [7]. M1 and M2 macrophages could originally reflect T helper 1 (Th1)/Th2 immune response [8]. M1 macrophages promote inflammation, whereas M2 cells are critical for inflammation resolution [9]. M1 cells can be induced by lipopolysaccharide (LPS) and Th1-secreted interferon- $\gamma$  (IFN- $\gamma$ ), inducing nonallergic immune responses through releasing tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and interleukin-1 $\beta$  (IL-1 $\beta$ ). M2 macrophages are mainly activated by Th2-secreted cytokines such as IL-4, inducing allergic immune responses and lung inflammation resolution and tissue repair through

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releasing IL-4, IL-13, and IL-10. M1 macrophages are highly expressed various surface markers such as CCR2 (chemokine-C receptor-2) and CD68 (leukocyte differentiation antigen 68), and highly expressed a variety of markers such as CX3CR1 (chemokine-X3C receptor-1) and CD163 [6,10,11]. A variety of cytokines, chemokines, and immune-regulatory cells dynamically interplay and affect the polarization and function of M1 and M2 cells, subsequently influencing the pathogenesis and progression of asthma [6]. Therefore, modulation of polarization of M1 and M2 cells through molecular intervention has therapeutic potential in the treatment of asthma.

As the first line of defense against pathogens in the body, the innate immune system identifies pathogen-associated molecular patterns (PAMPs) encoded by pathogens, primarily through pattern recognition receptors (PRRs). Among these PRRs, nucleotide-binding oligomerization domain-like receptors (NLRs) are a very important class. NLR protein 3 (NLRP3, also known as NALP3), a member of NLR family, can be activated by PAMPs and damage-associated molecular patterns. Activated NLRP3 couples adaptor protein apoptosis-associated specklike protein (ASC) and caspase-1 to form a multiprotein cytosolic complex, NLRP3 inflammasome. NLRP3 oligomerization and recruitment of ASC and procaspase-1 trigger autoactivation of caspase-1, leading to the secretion of pro-inflammatory cytokines, such as IL-1 $\beta$  and IL-18 [12,13]. NLRP3 inflammasome plays a crucial role in the innate immune system [14]. Recent studies have shown that the NLRP3 inflammasome is involved in several adaptive immune diseases including asthma [15–17].

However, there is no report about effects of NLRP3 inflammasome on the polarization of M2 or M2 macrophages. A published study reported that NLRP3 could promote the expression of IL-4 (M2 stimulus) in Th2 cells [16]. Therefore, we speculated that NLRP3 may drive the polarization of M2 macrophages by up-regulating IL-4 and participate in the regulation of macrophages in asthma. In the present study, we utilized human primary monocytes and monocyte-derived macrophages to study expression of NLRP3 inflammasome components (NLRP3, ASC, and caspase-1) and its downstream cytokine IL-1 $\beta$ . By gain- or loss-of-function assays, we next explored the effects of NLRP3 inflammasome on M1/M2 polarization and secretion of IL-4, IFN- $\gamma$ , TNF- $\alpha$ , and IL-1 $\beta$ . Then, we investigated whether IL-4 was involved in NLRP3-mediated regulation of M1/M2 proportion and clarified the mechanism by which NLRP3 up-regulated IL-4. Finally, we established allergic asthma model of mice to determine the *in vivo* functional role of NLRP3.

## Materials and methods

### Isolation of peripheral blood mononuclear cells, monocytes, and monocyte-derived macrophages

Peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats obtained from healthy blood donors ( $n=30$ ) and asthma patients ( $n=30$ ) obtained from the First Affiliated Hospital of Zhengzhou University by the density gradient separation technique using Ficoll-Hypaque (GE Lifesciences, U.S.A.). The asthma patients include 17 female and 13 male, ranging in age from 21 to 71 years, with mean age of  $54.53 \pm 6.34$ . Asthma was defined as physician-diagnosed asthma and a history of demonstrated airway hyper-reactivity to hypertonic saline (a provocative dose causing a 15% fall in forced expiratory volume in 1 s, 15 l) and/or a response to bronchodilator of 0.12% or 200 ml [18]. The healthy donors, nonsmokers with normal pulmonary function, were selected as a normal control group. The study was approved by the Ethics Committee of the First Affiliated Hospital of Zhengzhou University and written informed consent was obtained from participants. Positive selection of monocytes was conducted using CD14 MicroBeads (Miltenyi Biotec, China). CD14<sup>+</sup> monocytes were cultured for 7 days in RPMI 1640 complemented with 50 ng/ml macrophage colony-stimulating factor (Peprotech, U.S.A.).

### Macrophage polarization

After 7 days in culture, monocyte-derived macrophages from healthy blood donors were treated either with 100 ng/ml IFN- $\gamma$  + 100 ng/ml LPS (for M1 polarization) or with 20 ng/ml IL-4 (for M2 polarization) or with complete medium alone (control cells, M0) for 24 h. Cell lysates were collected for RNA and protein expression detection.

### Reverse transcription and quantitative PCR

Reverse transcription and quantitative PCR (RT-qPCR) was performed as described recently [9]. Total RNA from macrophages, transfected or not, treated or not, was isolated using the RNeasy mini kit including a

DNase treatment step (Qiagen, CA). RNA was reversed transcribed in the presence of 2.5 mM oligo (dT) using the Transcriptor High Fidelity cDNA Synthesis Kit (Roche, U.S.A.). Approximately 5 ng of cDNA was synthesized using the Mesa Blue qPCR MasterMix Plus for SYBR Assay (Eurogentec, Belgium) in the Light Cycler LC480 (Roche). mRNA expression was normalized to the levels of housekeeping gene ribosomal protein L13a (RPL13A, GenBank accession #NM\_012423.2). The relative level of expression of a gene was calculated using the  $2^{-\Delta\Delta C_t}$  method. Primers were listed in Supplementary Table S1.

## Western blot analysis

Total proteins from macrophages, transfected or not, treated or not, were lysed in (5×) Laemmli SDS buffer containing 0.01% bromophenol blue, 1%  $\beta$ -mercaptoethanol, and protease inhibitor cocktail (Roche). Subsequently, equal amounts of protein were separated by SDS–PAGE gels and transferred onto PVDF membranes (Millipore, U.S.A.). After blocking with 5% fat-free milk, the membranes were washed with TBS-T and incubated with primary and secondary antibodies according to the manufacturers' instructions. Primary antibodies against ASC (1 : 1000), caspase-1 (1 : 1000), and NLRP3 (1 : 1000), and IL-1 $\beta$  (1 : 500) were from Santa Cruz Biotechnology. Secondary HRP-linked antibodies against rabbit and mouse IgG (1 : 2000) and anti-GAPDH (1 : 5000) were from Cell Signaling. The protein was analyzed by an enhanced chemiluminescence kit (ECL kit, Pierce Biotechnology, IL), and band intensity was quantified with software Quantity One. GAPDH served as the loading control.

## Enzyme-linked immunosorbent assay

Cell culture supernatants from human macrophages were collected, centrifuged for 10 min at 250g and kept at  $-20^{\circ}\text{C}$  for cytokine measurements. The levels of various cytokines including IL-4, IL-1 $\beta$ , TNF- $\alpha$ , and IFN- $\gamma$  were measured with their commercial enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, U.S.A.) following the manufacturers' protocols.

## Small-interfering RNA transfection

The small-interfering RNA (siRNAs) specific for NLRP3, ASC, and caspase-1 were designed and synthesized by GenePharma (Shanghai, China). Macrophages were transfected with these siRNAs for 24 h through the use of Transit-TKO transfection reagent following the manufacturer's instructions (Mirus).

## Construction of plasmids and transfection

To overexpress NLRP3, the full-length NLRP3 cDNA fragment was cloned into pcDNA 3.1 vector (Invitrogen, U.S.A.), generating pcDNA3.1-NLRP3. Macrophages were transfected with pcDNA3.1-NLRP3 or empty pcDNA3.1 vector.

## Immunoblot and immunoprecipitation assays

Immunoblot and immunoprecipitation assays were performed as described recently [16]. In brief, cell lysates were prepared and then incubated with loading buffer (125 mM Tris–HCl, pH 6.8, 10%  $\beta$ -mercaptoethanol, 4.6% SDS, 20% glycerol, and 0.003% bromophenol blue). Subsequently, cell lysates were separated by SDS–PAGE and developed with specific primary and secondary antibodies

## Luciferase reporter assay

Luciferase reporter assay was performed as described recently [16]. The sequence encoding NLRP3 was inserted into the plasmid PGEX-4T1 (GE Healthcare Life Sciences, U.S.A.). The sequence encoding IRF4 was inserted into plasmid p3XFLAG-CMV-10 (Sigma–Aldrich, U.S.A.). The pIL-4 luciferase construct was obtained by inserting 3444-base pair fragments of the mouse IL-4 promoter into the multi-cloning site of the vector pGL3-Basic (Promega). Fragments were amplified by high-fidelity PCR with mouse genomic DNA as the template and specific primers. Human HEK293T cells were transiently transfected for 48 h with the reporter plasmid and the plasmid pRL-TK (Promega) through Lipofectamine 2000 (Invitrogen, U.S.A.). Luciferase was measured with the Dual Glo Luciferase Assay System following the manufacturer's instructions (Promega). Dual Glo Luciferase Reagent was added to the cells. After 10 min of incubation, firefly luciferase activity was measured with a Wallac 1440 Victor2 luminometer (PerkinElmer). The reaction was stopped by the addition of Dual-Glo Stop and Glo Reagent for 10 min and renilla luciferase activity was then measured.

## Animals

Twenty male BALB/c mice (4–6 weeks old, weighing  $20 \pm 29$  g) were purchased from Shanghai SLAC Laboratory Animal Co., Ltd (Shanghai, China). The present study was approved by the Animal Ethics Committee of the First Affiliated Hospital of Zhengzhou University. All experimental procedures were performed in strict accordance with the guidelines for the Care and Use of Laboratory Animals of the National Institutes of Health. Mice were randomly divided into four groups: normal control group, asthma model group, siRNA Ctrl group, and NLRP3 siRNA (si-NLRP3) group ( $n = 10$  for each group). Mice in asthma, siRNA Ctrl, and si-NLRP3 siRNA groups were sensitized by an intraperitoneal (i.p.) injection of 10  $\mu$ g of ovalbumin (OVA), and emulsified in 20 mg Al(OH)<sub>3</sub> gel in 0.1 ml of normal saline. Normal control group was injected (i.p.) with normal saline. On the 26th–29th day, mice in the siRNA Ctrl group and the si-NLRP3 group were challenged intranasally with concentrated siRNA (100  $\mu$ g); control mice were challenged intranasally with saline; asthma mice was challenged intranasally with PBS. On the 28th–30th day, control mice were given intranasal nasal drops. Mice in asthma, siRNA Ctrl, and si-NLRP3 groups were challenged intranasally with OVA solution (50  $\mu$ g). Mice were killed at 12 h after the final intranasal injection.

## Bronchoalveolar lavage

Bronchoalveolar lavage was performed by washing the lungs four times with 0.5 ml of saline at room temperature. After centrifugation at 400g for 10 min at 4°C, the cell-free bronchoalveolar lavage fluid (supernatant) was collected and stored at –20°C for cytokine analysis.

## Lung histology

Lungs separated from mice were stained with hematoxylin and eosin (HE) for histopathological analysis. In brief, fresh tissues were fixed in 4% paraformaldehyde solutions, and 3- $\mu$ m thick sections were stained with HE following the routine staining procedure and were examined with a microscope (Nikon E100; Nikon Corp., Japan). Infiltrates were assessed by a semi-quantitative score (0–3) by two observers independently.

## Statistical analysis

All statistical analyses were performed using SPSS version 18.0 software (IBM). Statistical differences between two independent groups were determined using *t*-test. The data are presented as the mean  $\pm$  SD from three independent experiments. Values of  $P < 0.05$  were considered statistically significant.

## Results

### Association of asthma with an M1/M2 ratio

PBMCs were isolated from peripheral venous blood of normal volunteers and asthma patients. Flow cytometry was performed to detect the proportion of peripheral blood M1 monocytes and M2 monocytes. As shown in Figure 1A,B, proportions of both M1 and M2 monocytes in peripheral blood of asthma patients were higher, while the M1/M2 ratio was lower than the normal control group.

### Association of asthma with NLRP3 inflammasome

To investigate whether NLRP3 inflammasome involved in asthma, qRT-PCR and Western blot were performed to evaluate mRNA and protein levels of NLRP3 inflammasome components (NLRP3, ACS, and caspase-1) and its downstream cytokine IL-1 $\beta$ . As shown in Figure 1C,D, expression of NLRP3, ACS, and caspase-1 in isolated macrophages of asthma patients were significantly higher than that of the normal control group, indicating that NLRP3 inflammasome was activated in asthma. Furthermore, asthma showed a higher level of pro-inflammatory cytokine IL-1 $\beta$  compared with the normal controls. These results suggested that NLRP3 inflammasome was activated and involved in the inflammation of asthma.

### Expression of NLRP3 inflammasome and IL-1 $\beta$ in M0/M1/M2 macrophages

After 7 days in culture, macrophages derived from monocytes of healthy blood donors were polarized toward an M1, M2, or M0 phenotype (resting macrophages) for 24 h. Cell lysates were collected for RNA and protein expression detection. Data revealed that compared with M0 macrophages, NLRP3 expression was markedly up-regulated in M1 macrophages and down-regulated in M2 macrophages, whereas caspase-1 expression was

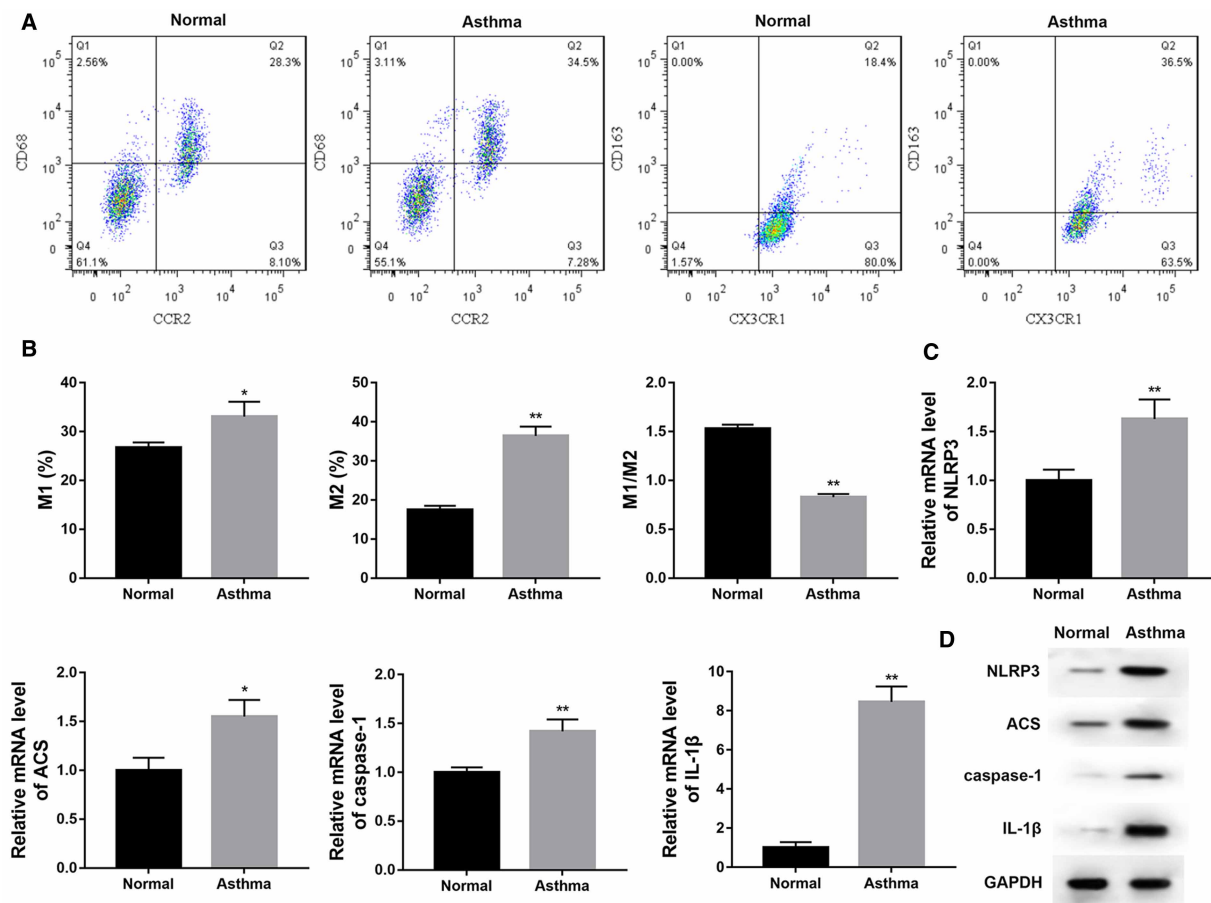


**Figure 1. Association of asthma with M1/M2 ratio and NLRP3 inflammasome.**

(A,B) PBMCs were isolated from peripheral venous blood of normal volunteers ( $n = 30$ ) and asthma patients ( $n = 30$ ). Flow cytometry was performed to detect the proportions of peripheral blood M1 monocytes and M2 monocytes. The labeled antibodies of M1 monocytes contained CCR2 and CD68, and M2 monocytes were labeled with CX3CR1 and CD163. Then, M1/M2 ratio was calculated. (C,D) RT-PCR and Western blot were performed to evaluate mRNA and protein levels of NLRP3, ACS, caspase-1, and IL-1 $\beta$  in isolated macrophages. \* $P < 0.05$ , \*\* $P < 0.01$ , vs. normal. GAPDH served as the loading control.

significantly lower in M2 macrophages. However, there was no significant change of ASC expression among M0/M1/M2 macrophages. IL-1 $\beta$  expression showed a similar pattern with NLRP3 (Figure 2).

## Silence of NLRP3 down-regulated IL-4 and up-regulated M1/M2

To investigate the role of NLRP3, ASC, and caspase-1 in the secretion of IL-4 (M2 stimulus), IFN- $\gamma$  (M1 stimulus), TNF- $\alpha$ , and IL-1 $\beta$  as well as M1/M2 ratio, we transfected macrophages from healthy donors and asthma patients with si-NLRP3, si-ASC, si-caspase-1, and control siRNA. The knockdown efficiencies of NLRP3, ACS, and caspase-1 were confirmed by Western blot (Figure 3A). Compared with the control siRNA-transfected group, silence of NLRP3 significantly down-regulated IL-4 in both asthmatic and normal control macrophages (Figure 3B). As for M1/M2 proportion, silence of NLRP3 did not affect M1, but obviously down-regulated M2, thereby up-regulating M1/M2 ratio (Figure 4). Furthermore, silence of NLRP3, ASC, and caspase-1 significantly down-regulated IL-1 $\beta$  compared with the control siRNA-transfected group. However, silence of ASC and caspase-1 had no significant effect on the secretion of IL-4, IFN- $\gamma$ , and TNF- $\alpha$  as well as the percentage of M1/M2 macrophages (Figures 3B and 4).

## Overexpression of NLRP3 up-regulated IL-4 and down-regulated M1/M2

To further document the effect of NLRP3 on the secretion of IL-4, IFN- $\gamma$ , TNF- $\alpha$ , and IL-1 $\beta$  as well as M1/M2 ratio, we transfected macrophages from healthy donors and asthma patients with

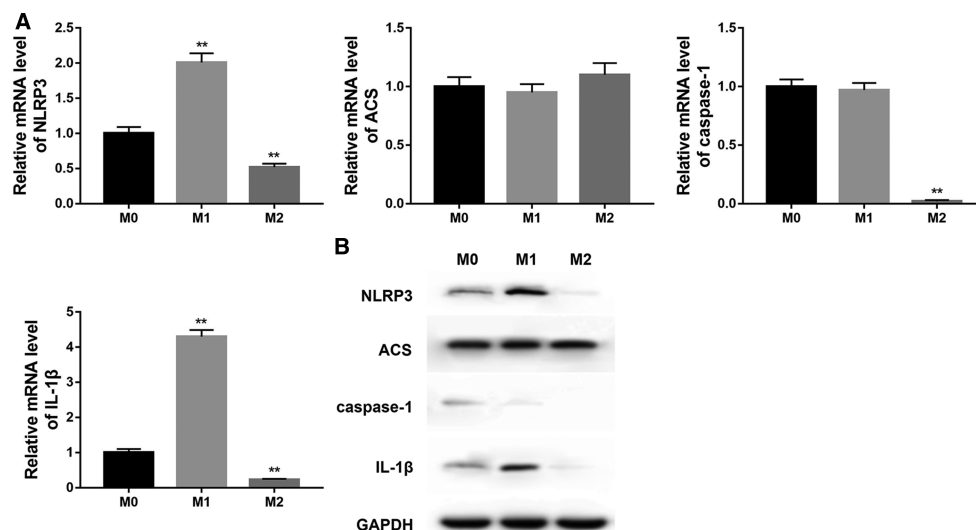


**Figure 2. Expression of NLRP3 inflammasome and IL-1β in M0/M1/M2 macrophages.**

After 7 days in culture, macrophages derived from monocytes of healthy blood donors were polarized toward M0 (complete medium alone), M1 (100 ng/ml IFN-γ + 100 ng/ml LPS), and M2 (20 ng/ml IL-4) macrophages for 24 h. Cell lysates were collected for RNA and protein expression detection. (A) RT-qPCR was performed to evaluate mRNA levels of NLRP3, ACS, caspase-1, and IL-1β in M0/M1/M2 macrophages. (B) Western blot analysis of protein levels of NLRP3, ACS, caspase-1, and IL-1β in M0/M1/M2 macrophages. \*\* $P < 0.01$  vs. M0. GAPDH served as the loading control.

pcDNA3.1-NLRP3 and control pcDNA3.1 empty vector. The overexpression efficiency of NLRP3 was confirmed by Western blot (Figure 5E). We found that, compared with the control pcDNA3.1-transfected group, overexpression of NLRP3 did not affect M1 (Figure 5A), but greatly up-regulated M2 (Figure 5B), thereby down-regulating the ratio of M1/M2 (Figure 5C). In addition, NLRP3 overexpression significantly up-regulated IL-4 and IL-1β, but had no significant effect on the secretion of IFN-γ and TNF-α (Figure 5D).

### IL-4 was involved in NLRP3-mediated down-regulation of M1/M2 ratio

It was known that IL-4 enables to induce the production of M2 macrophages. We have just confirmed that NLRP3 overexpression up-regulated the secretion of IL-4. Therefore, we hypothesized that NLRP3 up-regulated IL-4, thereby increasing M2 and diminishing the M1/M2 ratio. We used an anti-IL-4-neutralizing antibody to pretreat asthmatic macrophages transfected with NLRP3 overexpression and control vector to detect its effect on the proportions of M1 and M2 macrophages. Data showed that anti-IL-4 significantly down-regulated M2 and up-regulated M1/M2. Moreover, anti-IL-4 blocked NLRP3 overexpression-mediated elevation of M2 and reduction in M1/M2 ratio (Figure 6A–C). However, NLRP3 overexpression did not affect the secretion of IFN-γ and TNF-α, but significantly up-regulated IL-1β. Furthermore, anti-IL-4 did not affect the secretion of IFN-γ and TNF-α, and IL-1β (Figure 6D). These results suggested that IL-4 was involved in NLRP3-mediated up-regulation of M2 proportion and down-regulation of M1/M2 ratio.

### NLRP3 interacted with IRF4 and was required for optimal IRF4-dependent IL-4 transcription

As reported before [16], NLRP3 bound the IL-4 promoter and transactivated it in conjunction with the transcription factor IRF4 in Th2 cells. Hence, to clarify the mechanism by which NLRP3 up-regulated IL-4 in macrophages, we first explored whether NLRP3 was able to directly enhance the ability of IRF4 by immunoprecipitation. Data revealed that NLRP3 interacted with IRF4 directly in isolated macrophages from healthy donors and asthma patients (Figure 7A). Then, luciferase assay was performed. A fragment of 3444 base pairs of the IL-4 promoter were cloned into a luciferase reporter plasmid. We observed that overexpression of IRF4 or NLRP3 alone in normal macrophages did not significantly induce transactivation of the IL-4 promoter

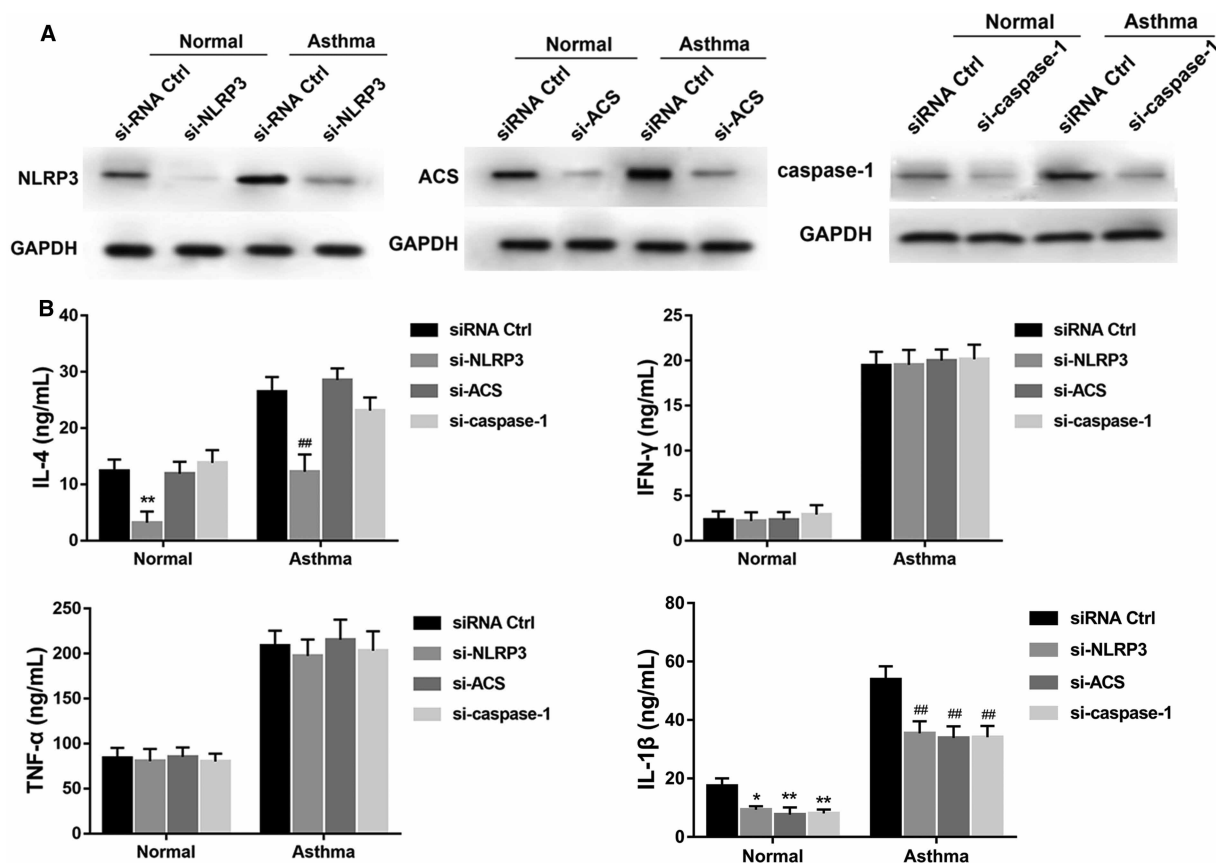


**Figure 3. Silencing of NLRP3 down-regulated IL-4.**

(A) Macrophages from healthy donors and asthma patients were transfected with si-NLRP3, si-ASC, si-caspase-1, and control siRNA. The knockdown efficiencies of NLRP3, ACS, and caspase-1 were confirmed by Western blot. (B) ELISA was used to evaluate the effects of si-NLRP3, si-ASC, and si-caspase-1 on the secretion of IL-4, IFN-γ, TNF-α, and IL-1β. \**P* < 0.05, \*\**P* < 0.01 vs. siRNA Ctrl in normal. ##*P* < 0.01 vs. siRNA Ctrl in asthma. GAPDH served as the loading control.

(Figure 7B). In contrast, co-transfection of plasmids for the expression of both IRF4 and NLRP3 enhanced transactivation of the IL-4 promoter. These data indicated that the direct interaction between NLRP3 and IRF4 was required for optimal IRF4-dependent IL-4 transcription in macrophages.

## Effect of NLRP3 silencing on lung inflammation, M1/M2 ratio, and secretion of TNF-α, IL-1β, and IL-4

To determine the *in vivo* functional role of NLRP3 in allergic asthma, we sensitized male BALB/c mice with OVA/A1(OH)<sub>3</sub>. Mice were randomly divided into normal control, asthma, siRNA Ctrl, and si-NLRP3 groups. Mouse lung tissues were isolated, and the inflammatory cell infiltration and airway morphology were evaluated by HE staining. Microscopically, asthmatic mice sensitized with OVA/A1(OH)<sub>3</sub> showed a robust cell infiltration, mucus hypersecretion, and epithelium thickening in lung tissues compared with saline-treated control mice. In contrast, we observed a significant reduction in cell infiltration and mucus secretion in lungs of OVA/A1(OH)<sub>3</sub>-sensitized si-NLRP3 mice compared with siRNA Ctrl mice (Figure 8A). Bronchoalveolar lavage fluid was collected and the proportion of M1/M2 macrophages, TNF-α, IL-1β, and IL-4 levels was detected. As shown in Figure 8B–D, asthma mice displayed significantly increased M2 proportion, decreased the ratio of M1/M2, and markedly up-regulated levels of TNF-α, IL-1β, and IL-4. However, silence of NLRP3 by si-NLRP3 dramatically down-regulated M2 and up-regulated M1/M2 ratio. Additionally, si-NLRP3 diminished IL-4 and IL-1β, but had little effect on the secretion of TNF-α.

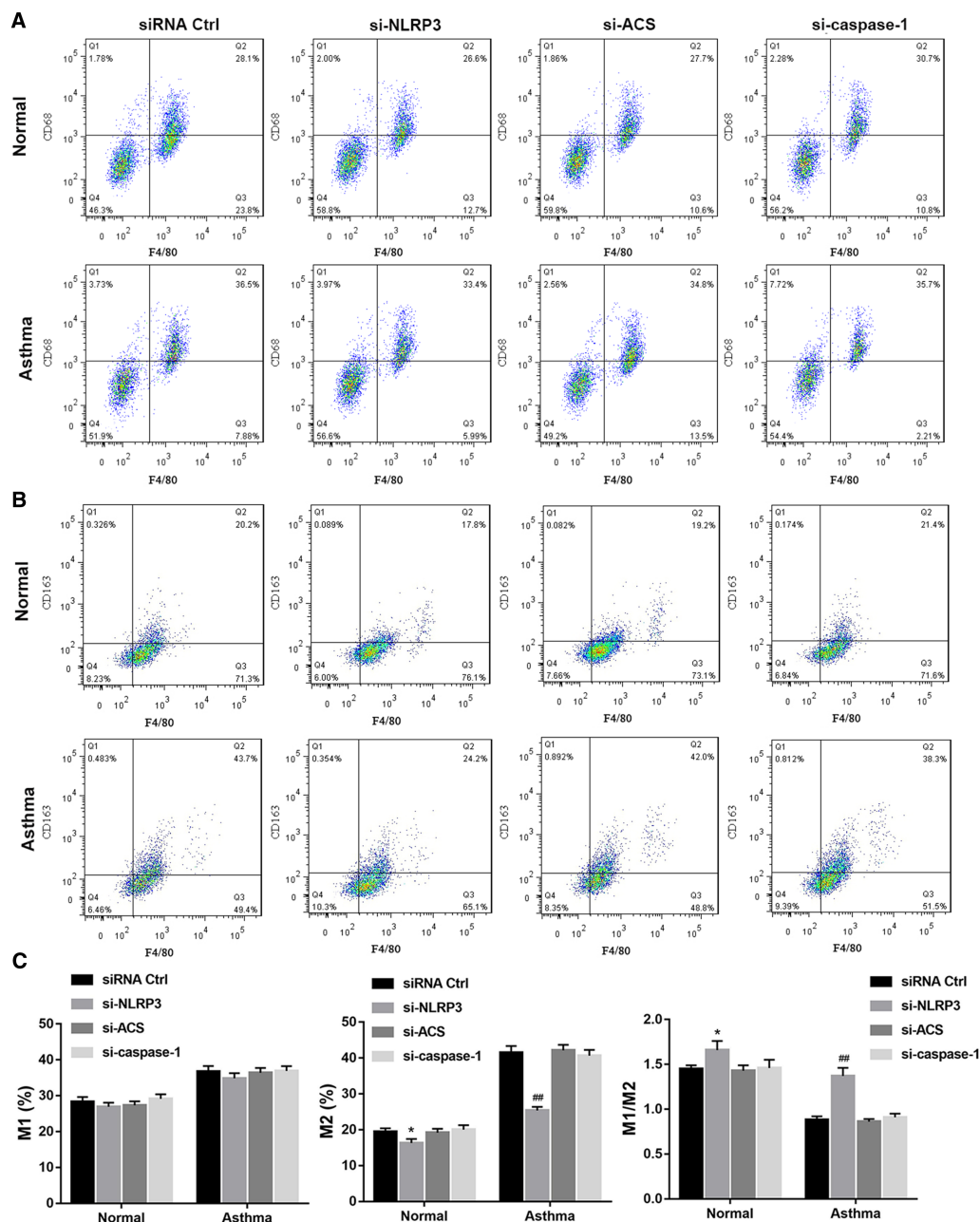


**Figure 4. Silence of NLRP3 up-regulated ratio of M1/M2.**

Flow cytometry was performed to detect the proportions of (A) M1 macrophages and (B) M2 macrophages derived from healthy donors and asthma patients following transfection with si-NLRP3, si-ACS, si-caspase-1, and control siRNA. The labeled antibodies of M1 macrophages contained F4/80 and CD68, and M2 macrophages were labeled with F4/80 and CD163. (C) Histograms of M1 and M2 proportion were shown and M1/M2 ratio was calculated. \* $P < 0.05$  vs. siRNA Ctrl in normal. ## $P < 0.01$  vs. siRNA Ctrl in asthma.

## Discussion

Asthma is a chronic inflammatory disorder of the airways caused by multiple genetic and environmental factors [19]. To date, the pathogenesis of asthma is still incompletely understood. There are several distinct forms of asthma: allergic, non-allergic, and intrinsic type. These forms can coexist in some patients. Clinically, the major focus of treatment and research has been on allergic asthma, the most common asthma phenotype [20].

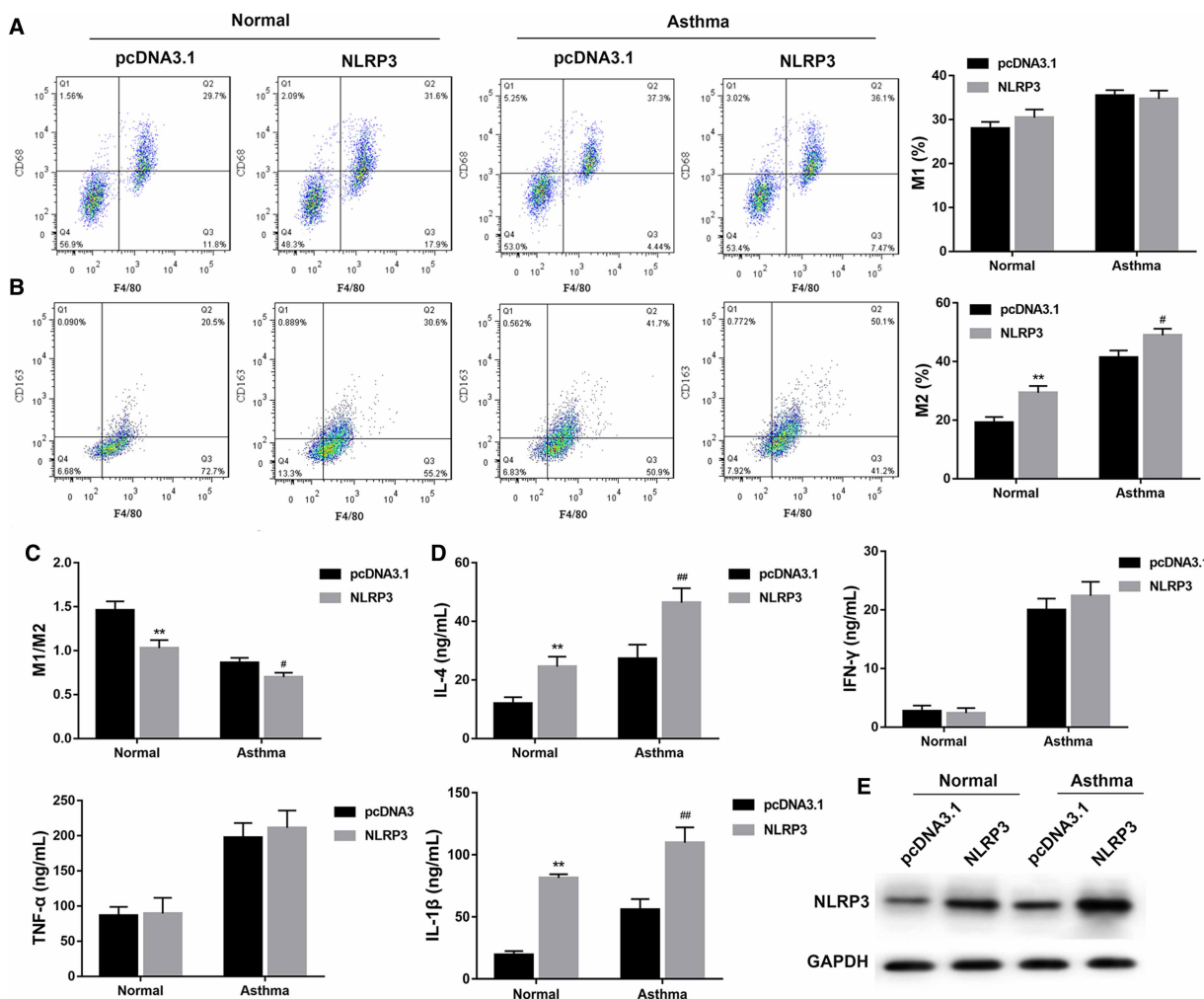


**Figure 5. Overexpression of NLRP3 up-regulated IL-4 and down-regulated M1/M2 ratio.**

Flow cytometry was performed to detect the proportions of (A) M1 macrophages and (B) M2 macrophages derived from healthy donors and asthma patients following transfection with pcDNA3.1-NLRP3 and control pcDNA3.1 empty vector. (C) M1/M2 ratio was calculated. (D) ELISA was used to evaluate the effect of pcDNA3.1-NLRP3 on the secretion of IL-4, IFN- $\gamma$ , TNF- $\alpha$ , and IL-1 $\beta$ . (E) The overexpression efficiency of NLRP3 was confirmed by Western blot. \*\* $P < 0.01$  vs. pcDNA3.1 in normal. # $P < 0.05$ , ## $P < 0.01$  vs. pcDNA3.1 in asthma. GAPDH served as the loading control.

Many cell types have been implicated in the pathogenesis of asthma. The contributions of T helper 1 (Th1) cells, Th2 cells, Th17 cells, type 2 innate lymphoid, dendritic cells, and mast cell and eosinophil activation have been documented [21,22]. Among these, the imbalance of Th1/Th2 was observed in asthma patients [23], and the dominance of Th2 cells is closely related to the incidence of asthma [24]. However, macrophages, a major airway immunocyte, have been increasingly understood to contribute to allergic inflammation in asthma [21,22,25–29]. Macrophages participate in lung immune homeostasis through phagocytosis and the release of mediators in response to antigens [30,31]. The polarization of macrophages indicates that pro-inflammatory responses associated with M1 phenotype or anti-inflammatory responses associated with M2 phenotypes dominate under specific conditions in their microenvironment, at any given time point [32]. M1 macrophages promote extracellular matrix degradation and tissue injury, whereas M2 macrophages are involved in control of collagen production and tissue remodeling [33,34]. M2 cells can be divided into M2a, M2b, and M2c cells. M2a cells can be activated by IL-4, IL-13, and IL-33, inducing allergic immune responses through releasing IL-4 and IL-13. M2c cells can be activated by IL-10 and transforming growth factor- $\beta$ , inducing lung inflammation resolution and tissue repair through releasing IL-10 [6]. However, identification of these M2 subtypes is not well clarified in asthma of human subjects and animal models [35,36]. In a word, M1 and M2 cells are

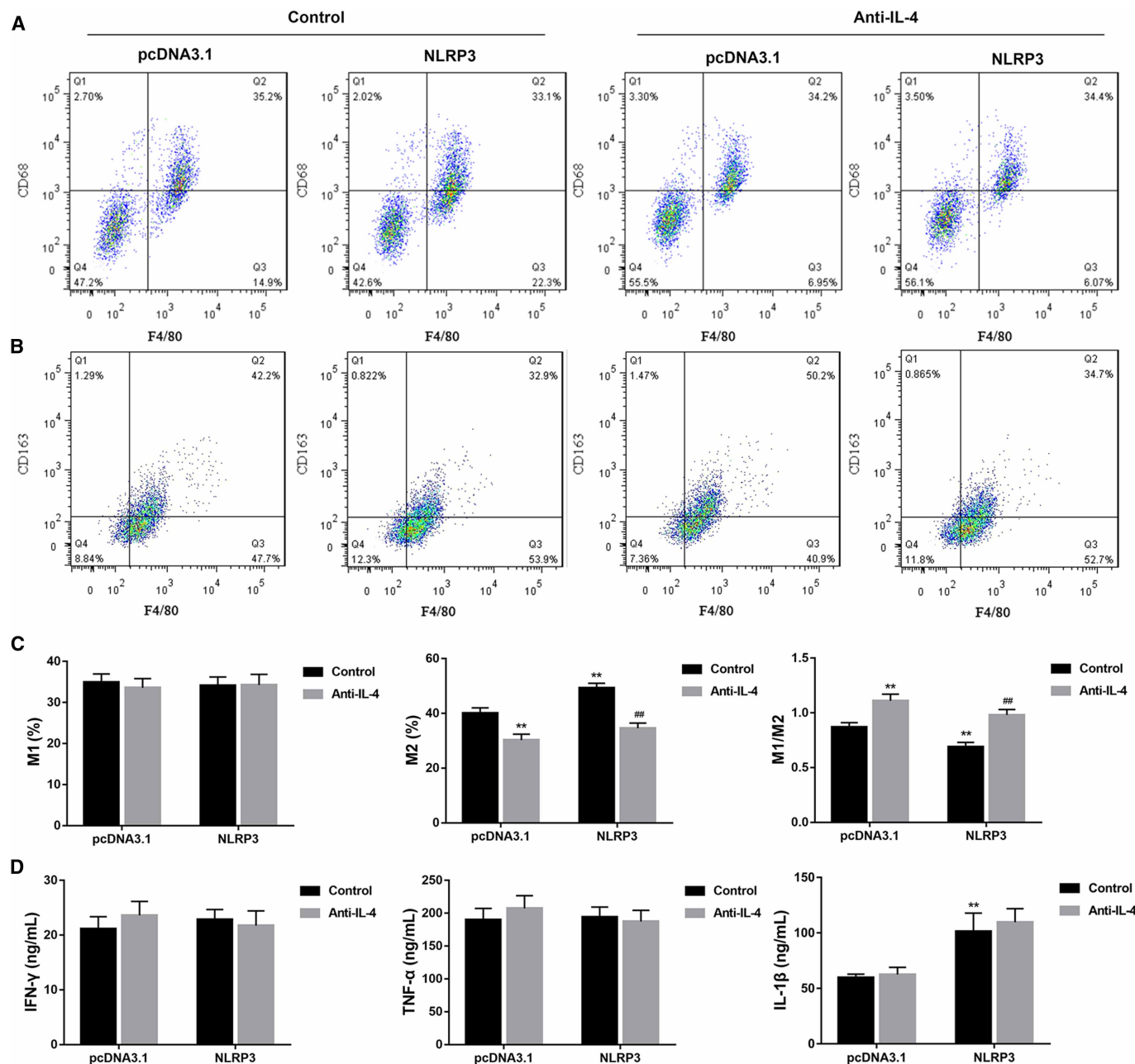


**Figure 6. IL-4 was involved in NLRP3-mediated down-regulation of M1/M2 ratio.**

Flow cytometry was performed to detect the proportions of (A) M1 macrophages and (B) M2 macrophages derived from asthma patients following transfection with pcDNA3.1-NLRP3 and control pcDNA3.1 empty vector in the presence of anti-IL-4 or not. (C) M1/M2 ratio was calculated. (D) ELISA was used to evaluate the effect of anti-IL-4 and NLRP3 overexpression on the secretion of IFN- $\gamma$ , TNF- $\alpha$ , and IL-1 $\beta$ . \*\* $P$  < 0.01 vs. control in pcDNA3.1. ## $P$  < 0.01 vs. control in NLRP3.

differently implicated in the initiation and resolution of lung inflammation during allergic immune responses [6]. Owing to the distinct role of M1 and M2 cells in the pathogenesis of asthma, it is important to maintain an optimal balance between the population of M1 and M2 cells. As a result, modulation of polarization of M1 and M2 macrophages has therapeutic potential in asthma [37].

M2 macrophages differentiate under control of Th2 immune processes. Activation of M2 macrophages is correlated with multiple immune pathways that influence adaptive immune responses and promote allergic

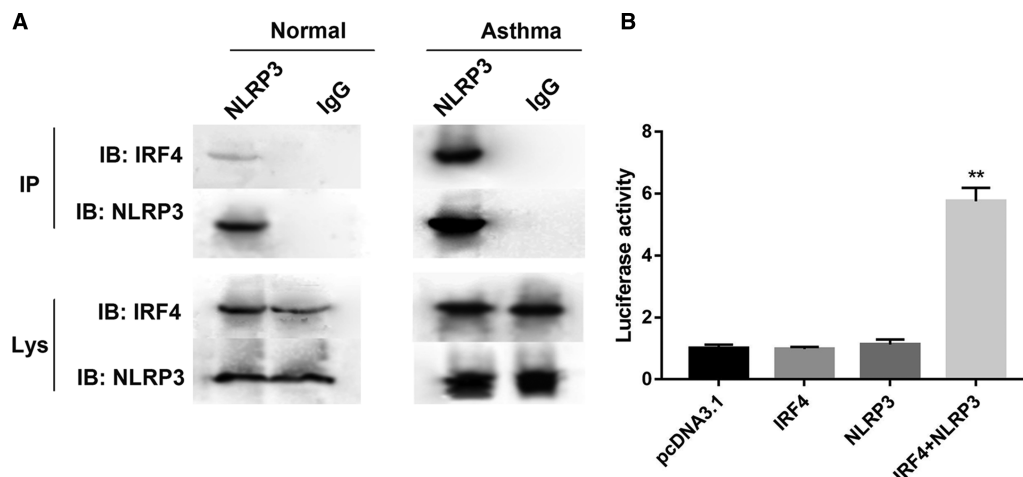


**Figure 7. NLRP3 interacted with IRF4 and was required for optimal IRF4-dependent IL-4 transcription.**

(A) Immunoblot analysis (IB) of IRF4 or NLRP3 in lysates of macrophages from normal healthy donors and asthma patients, assessed after immunoprecipitation (IP) with anti-NLRP3 or immunoglobulin control (IgG), or without immunoprecipitation [bottom (Lys)]. (B) Luciferase activity in normal macrophages transfected with an IL-4 promoter-driven luciferase reporter along with plasmid for the expression of IRF4 and/or NLRP3.

airway inflammation. The abundance of M2 cells is linked to declines in lung function [38]. A previous study reported that M2 phenotype (CD206<sup>high</sup>MHC-II<sup>high</sup>) alveolar macrophages were increased in bronchoalveolar lavage fluid obtained from asthma patients compared with healthy subjects [38]. In the current study, we found that compared with normal healthy controls, asthma patients showed higher proportions of both M1 and M2 monocytes in peripheral blood, but lower M1/M2 ratio indicating the dominance of M2 cells. We also observed a robust cell infiltration and mucus hypersecretion in OVA/A1(OH)<sub>3</sub>-induced asthmatic mice. These findings revealed that increased numbers of M2 cells might reflect greater disease severity and contribute to the pathobiology of asthma.

The activation of NLRP3 inflammasome in the lung can be triggered by stimuli such as infections, allergens, cigarette smoke, and airway pollutants in the asthmatic airway [39]. Bioactive IL-1 $\beta$  is one of the main terminal products generated by activation of the inflammasome. NLRP3 inflammasome and concomitant inflammatory mediator IL-1 $\beta$  contribute to the development of inflammation, mucus hypersecretion, and airway hyper-responsiveness [39]. The activated NLRP3 inflammasome is involved in the pathogenesis of chronic obstructive pulmonary disease, respiratory infections, and neutrophilic asthma [40–42]. A published study revealed that NLRP3 inflammasome is up-regulated in neutrophilic asthma and may regulate the inflammation process observed in this asthma phenotype through the production of IL-1 $\beta$  [18]. Consistent with this previous study, we observed that expression of NLRP3 inflammasome components (NLRP3, ACS, and caspase-1) and its downstream pro-inflammatory cytokine IL-1 $\beta$  was significantly higher in isolated macrophages of asthma patients than that of the normal control group. Our findings indicated that NLRP3 inflammasome was activated and involved in the inflammation of asthma. Furthermore, expression of NLRP3 and IL-1 $\beta$  was markedly up-regulated in M1 macrophages and down-regulated in M2 macrophages compared with M0 cells. We also found that only NLRP3 in NLRP3 inflammasome could promote the polarization of M2 macrophages and the secretion of IL-4 (M2 stimulus). However, NLRP3 had no significant effect on M1 proportion and the secretion of IFN- $\gamma$  (M1 stimulus) and TNF- $\alpha$ . IL-4 is a key cytokine in asthma pathophysiology, alternative macrophage (M2 cells) activation, and its receptor is a new pharmacological target in severe asthma [43]. Given the results obtained above, we thus used anti-IL-4 to explore whether NLRP3 may drive M2 cell-biased polarization through IL-4 up-regulation. Data confirmed that anti-IL-4 blocked NLRP3 overexpression-mediated up-regulation of M2 proportion and down-regulation of M1/M2 ratio, which confirmed our hypothesis. Moreover, consistent with prior observations before [16], we confirmed that NLRP3 up-regulated IL-4 expression through an interaction with IRF4 in macrophages.

The role of NLRP3 inflammasome in asthma remains to be elucidated. Several studies have observed the role of the NLRP3 inflammasome in the development of allergic airway inflammation in both an adjuvant-

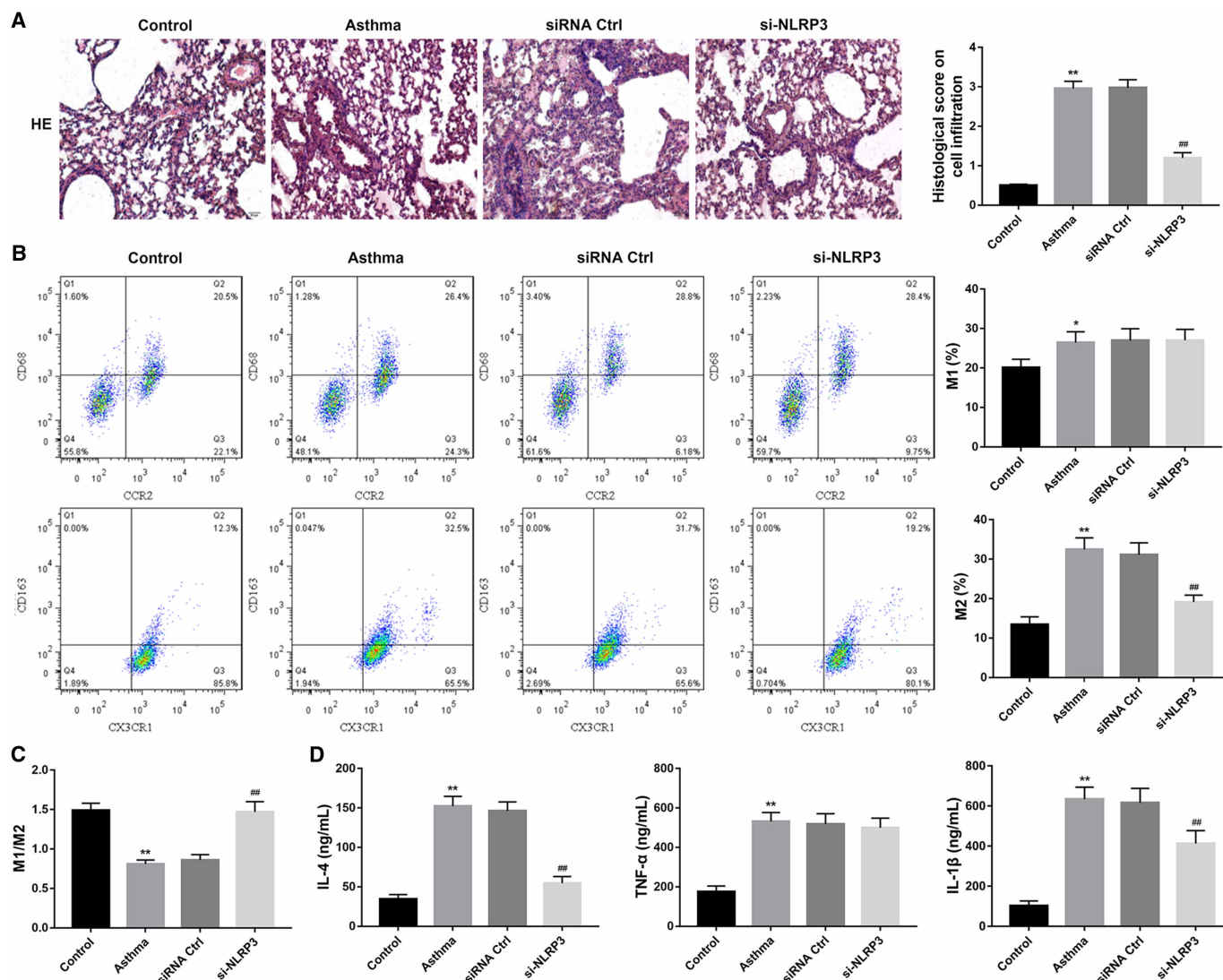


**Figure 8. Effect of NLRP3 silencing on lung inflammation, M1/M2 ratio, and secretion of IL-4, TNF- $\alpha$ , and IL-1 $\beta$  in asthma mice.**

(A) Mouse lung tissues were isolated and the inflammatory cell infiltration and airway morphology were evaluated by HE staining. The extent of peribronchial and perivascular inflammation was scored by two independent observers. (B) Bronchoalveolar lavage fluid was collected and proportions of M1 and M2 macrophages were detected. (C) Then M1/M2 ratio was calculated. (D) TNF- $\alpha$ , IL-1 $\beta$ , and IL-4 levels in bronchoalveolar lavage fluid were determined. \* $P < 0.05$ , \*\* $P < 0.01$  vs. control. ## $P < 0.01$  vs. siRNA Ctrl.

dependent model of allergic asthma (OVA injected along with the adjuvant alum) [44] and an adjuvant-free model (injection of OVA alone) [45]. A recent study showed *in vivo* that NLRP3-mediated IL-1 $\beta$  responses play a critical role in OVA-induced allergic airway inflammation. NLRP3-knockdown mice exhibited marked reduction in the production of OVA-induced, Th2-related cytokines [15]. Consistent with published studies, in our adjuvant-dependent OVA-induced asthmatic model, NLRP3-knockdown mice showed less accumulation of mucus and less infiltration of eosinophils and lymphocytes into the lung tissues compared with saline-treated mice. Our findings demonstrated that deficiency of NLRP3 impaired lung inflammation in OVA-induced allergic asthmatic mice. Furthermore, we confirmed *in vivo* that, in bronchoalveolar lavage fluid, knockdown of NLRP3 decreased the population of M2 macrophages and secretion of IL-4 and IL-1 $\beta$ , and had no significant effect on M1 proportion and secretion of IFN- $\gamma$  and TNF- $\alpha$ .

Th1 cytokines, e.g. IFN- $\gamma$ , can stimulate monocytes to differentiate into M1 macrophages, and exposure to Th2 cytokines, e.g. IL-4, monocytes can differentiate into M2 macrophages. M2 macrophages were reported to

produce CCL17 to induce Th2 cells [46]. Furthermore, Braga et al. [47] proposed that in renal fibrogenesis, M2 macrophages were recruited and activated in a Th2 cell-prone inflammatory milieu, and MyD88 signaling is involved in renal fibrosis by favoring a Th2 immune response and activating M2 macrophages. Bruchard et al. [16] reported a very similar role for NLRP3 activation in the induction of Th2 cells which can also promote asthma like symptoms. They reported that in Th2 cells, NLRP3 bound the IL-4 promoter and transactivated it in conjunction with the transcription factor IRF4, and they also demonstrated the ability of NLRP3 to act as a key transcription factor in Th2 differentiation. Based on the above-mentioned studies, we speculated that the drive of the polarization of M2 macrophages by NLRP3 might support the differentiation of Th2 cells in asthma. However, further studies concerning this speculation are required.

## Conclusion

In summary, we demonstrated here that activation of NLRP3 was engaged in the promotion of asthma. NLRP3, functioned independently of the inflammasome, promoted the polarization of M2 macrophages through up-regulating the expression of IL-4, thereby contributing to its regulation of asthma.

## Abbreviations

ASC, apoptosis-associated specklike protein; CCR2, chemokine-C receptor-2; CD68, leukocyte differentiation antigen 68; CX3CR1, chemokine-X3C receptor-1; ELISA, enzyme-linked immunosorbent assay; HE, hematoxylin and eosin; IFN- $\gamma$ , interferon- $\gamma$ ; IL, interleukin; IL-1 $\beta$ , interleukin-1 $\beta$ ; LPS, lipopolysaccharide; NLR, (Nod)-like receptor; NLRP3, (Nod)-like receptor protein 3; OVA, ovalbumin; PAMPs, pathogen-associated molecular patterns; PBMCs, peripheral blood mononuclear cells; si-NLRP3, NLRP3 siRNA; Th1, T helper 1; TNF, tumor necrosis factor.

## Author Contribution

Y.L. conceived and designed the analysis. Y.L., X.G., Y.M., Y.W., H.W., Z.C., X.W., X.J., L.J., L.D., M.L., and L.A. collected the data. Y.L. and X.G. contributed data or analysis tools. Y.L., X.G. and H.W. performed the analysis. Y.L. wrote the paper.

## Competing Interests

The Authors declare that there are no competing interests associated with the manuscript.

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**Supplementary Table 1 Primers for RT-qPCR**

| <b>Genes</b> | <b>Forward</b>             | <b>Reverse</b>            |
|--------------|----------------------------|---------------------------|
| NLRP3        | GGAGAGACCTTTATGAGAAAGCAA   | GCTGTCTTCCTGGCATATCACA    |
| Caspase-1    | CCAGGACATTAAAATAAGGAAACTGT | CCAAAAACCTTTACAGAAGAATCTC |
| ASC          | TGACGGATGAGCAGTACCAG       | GCTTCCGCATCTTGCTTGG       |
| IL-1 $\beta$ | AGCTGATGGCCCTAAACAGA       | TCGGAGATTCGTAGCTGGAT      |