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ORIGINAL RESEARCH

ANXA3 regulates HIF1α-induced NLRP3 inflammasome activity and promotes LPS-induced inflammatory response in bronchial epithelial cells

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Abstract

Introduction: Childhood asthma is one of the most common pediatric diseases, and its incidence is increasing. Annexin A3 (ANXA3) is a member of the Annexin family, a well-known polygenic family of membrane binding proteins. Bioinformation analysis showed that ANXA3 was highly expressed in asthmatic patients, suggesting the effects of ANXA3 on asthma, whereas the mechanism is still unclear.

Methods: A inflammatory response model of bronchial epithelial BEAS-2B cells induced by LPS was constructed. Immunoblot and quantitative PCR assays were performed to detect the expression levels of ANXA3 in control or LPS-induced BEAS-2B cells. MTT, flow cytometry (FCM), and Immunoblot assays were respectively conducted to detect the effects of ANXA3 on survival and apoptosis of LPS-induced BEAS-2B cells. qPCR and ELISA assays were performed to detect the expression of TNF-α, IL-6, and IL-8. Additionally, Immunoblot assays were performed to detect the effects of ANXA3 on HIF1α and NLRP3 inflammasome in BEAS-2B cells.

Results: We found ANXA3 was overexpressed in LPS-induced BEAS-2B cells. ANXA3 ablation promoted the survival of LPS-induced BEAS-2B cells and suppressed the inflammatory response of LPS-induced BEAS-2B cells. Importantly, we noticed ANXA3 inhibited HIF1α-induced NLRP3 inflammasome activity, and increasing the expression of HIF-α rescued the effects of ANXA3 depletion on asthma.

Conclusion: ANXA3 enhanced LPS-triggered inflammation of human bronchial epithelial cells by regulating hypoxia-inducible factor-1α (HIF1α)-mediated NLRP3 inflammasome activation, and thought ANXA3 as a promising molecular target for acute asthma treatment.

Keywords

Annexin A3 (ANXA3); Acute asthma; Inflammatory response; NLRP3 inflammasome; Hypoxia-inducible factor-1α (HIF1α)

1. Introduction

Childhood asthma is one of the most common pediatric diseases, and its incidence is increasing year by year [1]. Acute asthma in children can be caused by viral diseases such as bronchiolitis and lung injury [2]. Acute asthma is characterized by airflow obstruction, persistent inflammation, and airway hyperresponsiveness (AHRs) to common ambient air allergens [3]. Inflammation is closely related to the pathogenesis of asthma, and the high levels of cytokines such as interleukin IL-4, IL-5, and IL-13 are main features of acute asthma [4]. Nucleotide binding domains and leucine-rich repeat protein 3 (NLRP3), a member of the NLP receptor family, mediates the formation of inflammasomes that play an important role in asthma [5]. Notably, NLRP3 inflammasome was activated in bronchial epithelial cells of asthmatic mice and increased the release of inflammatory factors.

Annexin A3 (ANXA3) is a member of the Annexin family, a well-known polygenic family of membrane binding proteins [6]. ANXA3 affected the cell proliferation, invasiveness, inflammation, and apoptosis in multiple tumors [7–9]. ANXA3 depletion improved intracranial aneurysms by inhibiting JNK pathway [10, 11]. In addition, ANXA3 ablation suppressed the proliferation and migration of pancreatic cancer cells [11]. Downregulation of ANXA3 could also alleviate pain associated bone cancer via VEGF [12]. Notably, the depletion of ANXA3 reduced inflammation in mice with lung injury, suggesting the effects of ANXA3 on the inflammation pathway [4]. Moreover, bioinformation analysis showed that ANXA3 was highly expressed in asthmatic patients, suggesting the effects of ANXA3 on asthma [13]. However, the mechanism is still unclear.

Previous study showed that ANXA3 positively regulated the

activity of hypoxia-inducible factor 1α (HIF1α), and inhibition of HIF1α attenuated inflammatory response in asthmatic mice [14]. HIF1α also promotes the activation of NLRP3 inflammasome and increases inflammatory response in bleomycin-induced acute lung injury [12]. Therefore, we hypothesized that the pro-inflammatory effect of ANXA3 is due to HIF1α-induced NLRP3 inflammasome activation.

In this study, we constructed an inflammatory response model of bronchial epithelial cells induced by LPS, and found that ANXA3 increased the inflammatory response of LPS-induced bronchial epithelial cells by regulating HIF1α-induced NLRP3 inflammasome activity. We therefore thought ANXA3 could serve as a promising therapeutic target for the treatment of acute asthma.

2. Materials and methods

2.1 Antibodies, primers and plasmids

β-actin (1 : 10000 dilution, A1978, Sigma), ANXA3 (1 : 1000 dilution, ab239582, Abcam), HIF1α (1 : 1000 dilution, ab179483, Abcam), Bax (1 : 1000 dilution, ab32503, Abcam), Bcl-2 (1 : 500 dilution, ab182858, Abcam), cleaved-caspase 3 (1 : 500 dilution, ab32042, Abcam), NLRP3 (1 : 1000 dilution, ab263899, Abcam), ASC (1 : 500 dilution, ab151700, Abcam), and Echinomycin (SML0477, Sigma-Aldrich) were used in this study.

The qPCR primer sequences of ANXA3 were: FP: 5′-TTAGCCCATCAGTGGATGCTG-3′, RP: 5′-CTGTGCATTTGACCTCTCAGT-3′. The qPCR primer sequences of TNF-α were: FP: 5′-CAGCCTCTTCTCCTTCCTGA-3′, RP: 5′-GGAAGACCCCTCCCAGATAGA-3′. The qPCR primer sequences of IL-6 were: FP: 5′-ATGAACTCCTTCTCCACAAGC-3′, RP: 5′-CTACATTTGCCGAAGAGCCCTCAGGCTGGACTG-3′. The qPCR primer sequences of IL-8 were: FP: 5′-ATGACTTCCAAGCTGGCCGTG-3′, RP: 5′-TTATGAATTCTCAGCCCTCTTCAAAAACTTCTC-3′. The qPCR primer sequences of GAPDH were as follows: FP: 5′-AACGGATTGGTCGTATTGGG-3′, RP: reverse, 5′-TCGCTCCTGGAAGATGGTGAT-3′.

Control or ANXA3 shRNA plasmids were all bought from Addgene.

2.2 Cell culture and treatment

The human bronchial epithelial BEAS-2B cells were bought from ATCC and maintained in DMEM supplemented with 10% of fetal bovine serum (FBS) and incubated at 37 °C in a 5% CO_2 incubator. To construct a LPS-induced cell model of inflammatory response, BEAS-2B cells were serum deprivation and treated with 25 μg/mL LPS (serotype 055 : B5, Sigma-Aldrich, St. Louis, MO) or Echinomycin (330175-M, Sigma-Aldrich, St. Louis, MO) for 24 h. ANXA3 shRNA plasmids (1 μg/μL) were transfected into cells by the use of Lipofectamine 2000 (11668019, Invitrogen, Thermo Fisher Scientific, Inc.) for 4 h. For 6-well plates, 5 μL transfection reagent and 1 μg plasmids were mixed in 200 μL of serum-free DMEM, left to stand for 5 min and then mixed. Following incubation at room temperature for 20 min, the mix was added to serum-starved cells and incubated at 37 °C for 4 h.

2.3 Immunoblot assay

Protein was quantified by BCA method, and 20 μg protein from BEAS-2B cells was added to each well. The cell samples were isolated to extract the proteins and separated by 10% SDS-PAGE, sequentially transferred onto the PVDF membranes for 2 h, and followed by blocking with 5% BSA in TBST buffer at room temperature for 2 h. PVDF membranes were subsequently incubated with primary antibodies targeted the indicated proteins at room temperature for 1.5 h. Subsequently the membranes were incubated with HRP-conjugated antibodies for 1 h. Signals were then visualized by the use of an ECL kit.

2.4 Quantitative PCR (qPCR) assays

Trizol (15596026, Invitrogen, USA) was used to extract all the RNA from BEAS-2B cells. Then RNA was reverse-transcribed by a type of M-MLV reverse transcriptase (M1701, Promega, USA). Total mRNA was reversely transcribed by the use of a cDNA synthesis system. Quantitative PCR was performed using a SYBR Ex Taq kit (638319, Takara, Japan), and the relative ANXA3 mRNA level was normalized to that of GAPDH.

2.5 MTT assay

BEAS-2B cells upon the indicated treatment were plated into the 96-well plates with a density of 800 cells per well. After 24 h culture, cells were subsequently treated with MTT agent for 4 h and washed with PBS. Cells were then isolated by the use of 200 μL DMSO and the OD value at 490 nm wavelength was analyzed.

2.6 Flow cytometry (FCM) assay

BEAS-2B cells were re-suspended and incubated with annexin V-FITC and propidium iodide (PI) at room temperature for 10 min. Subsequently the samples were analyzed by a FACS Calibur flow cytometer, and the apoptosis cells were analyzed and compared among different groups.

2.7 Enzyme linked-immunosorbent assay (ELISA)

BEAS-2B cell media was obtained for TNF-α (ab181421), IL-6 (ab178013) and IL-8 (ab214030) concentration measurement, according to the manufacturer's instructions (Abcam, Cambridge, UK). Cells were treated with the indicated drugs for 24 h, and then the ELISA assays were performed. Briefly, samples were added into detection wells. Then removed extra unbound solutions, biotin-conjugated specific antibodies were pipetted into each wells followed by addition of avidin conjugated Horseradish Peroxidase (HRP). After removal of any unbound avidin-enzyme reagent, substrate of the enzyme was added to the wells for color reaction. Stopped the reaction with correspondent buffer and measured the intensity of the color.

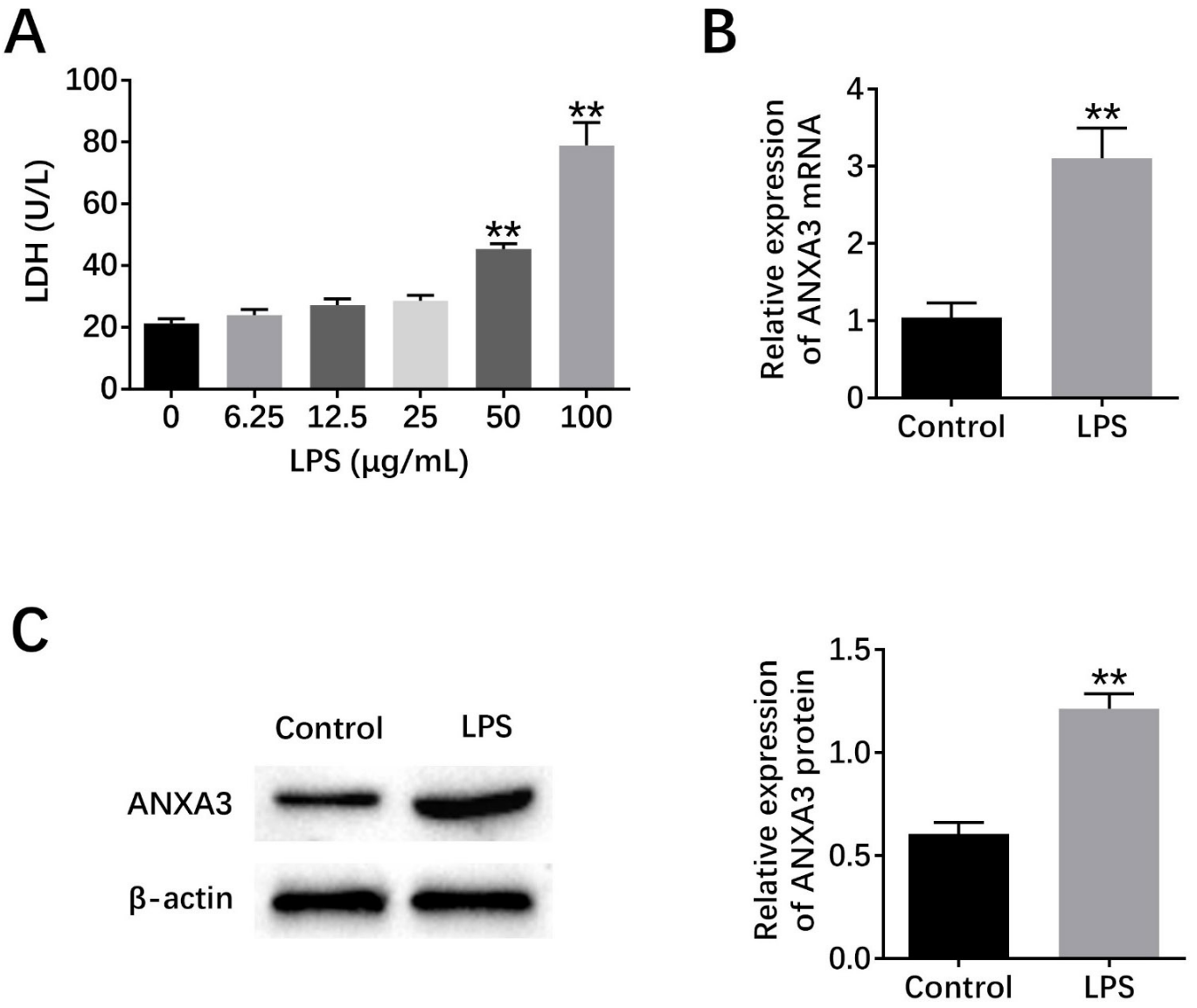


FIGURE 1. ANXA3 was overexpressed in LPS-induced BEAS-2B cells. (A) LDH leakage was confirmed after LPS treatment of BEAS-2B cells in a concentration-dependent manner. (B) Quantitative PCR assays showed the mRNA levels of ANXA3 in BEAS-2B cells upon the control or 25 μg/mL LPS treatment for 24 h. (C) Immunoblot showed the expression of ANXA3 in control or LPS-induced BEAS-2B cells. Results are presented as mean ± SEM, ** $p < 0.01$.

2.8 Statistics

GraphPad 7.0 software was used to perform statistical analysis. Data in this study were represented as mean ± SEM. Student's t-test was used for statistical comparisons and $p < 0.05$ was considered as statistically significant. * indicates $p < 0.05$, **$p < 0.01$, and ***$p < 0.001$, respectively.

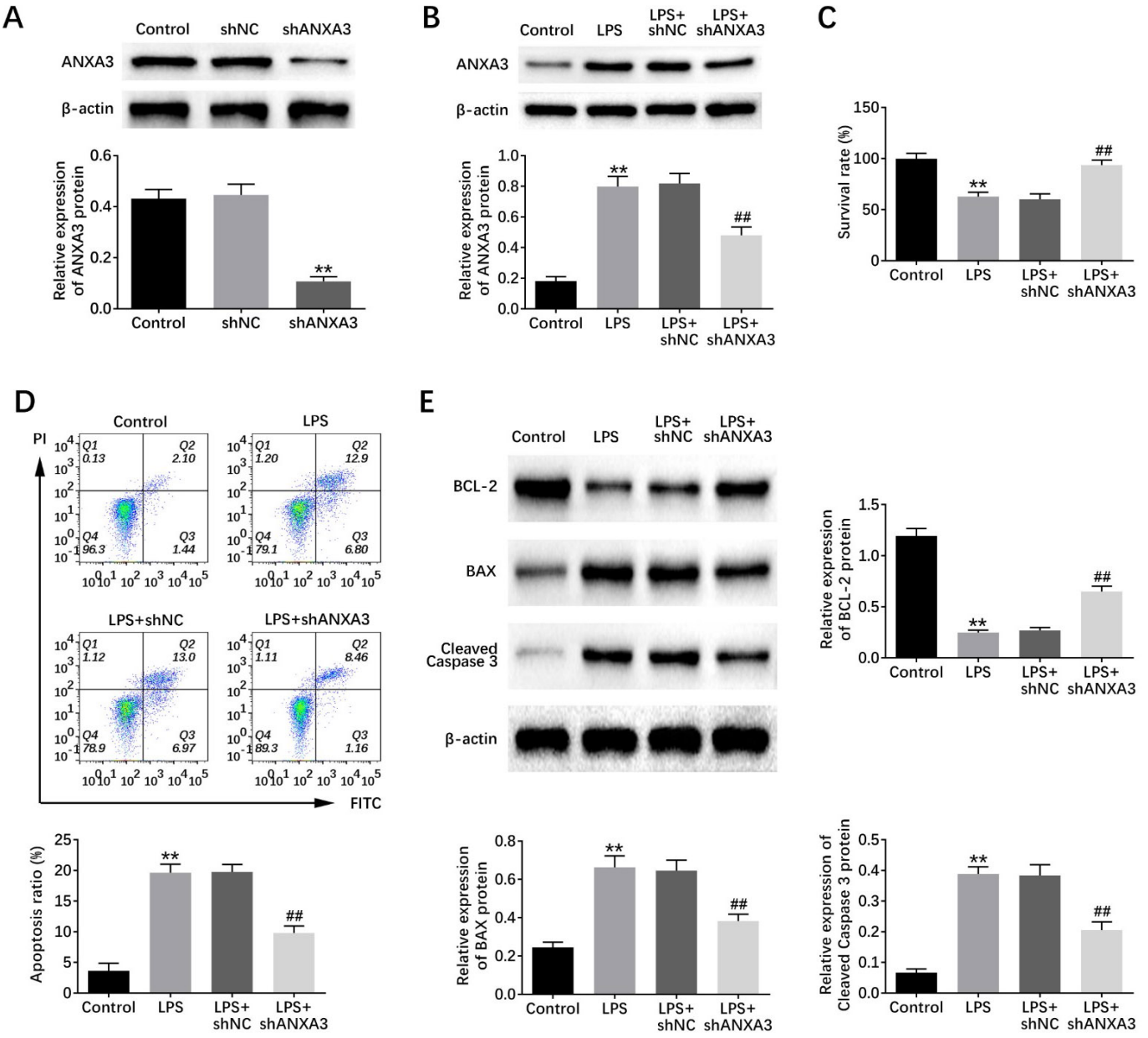
3. Results

3.1 ANXA3 was higher expression in LPS-induced BEAS-2B cells

To investigate the possible role of ANXA3 in the progression of asthma and asthma-related inflammation, we first constructed an LPS-induced inflammatory response model of bronchial epithelial BEAS-2B cells. By LDH leakage experiment, we noticed that the LDH release was obviously promoted after the treatment with LPS in BEAS-2B cells, in a concentration-dependent manner (Fig. 1A). Quantitative PCR assays showed the high mRNA levels of ANXA3 in LPS-induced BEAS-2B cells, compared with control cells (Fig. 1B). Similarly, immunoblot assays showed the higher ANXA3 expression in LPS-induced BEAS-2B cells (Fig. 1C). Collectively, these results indicated that ANXA3 was highly expressed in LPS-induced BEAS-2B cells.

3.2 ANXA3 knockdown promoted the survival and suppressed the apoptosis of LPS-induced BEAS-2B cells

We next investigated the effects of ANXA3 on the survival and apoptosis of LPS-induced BEAS-2B cells. We noticed that transfection of ANXA3 shRNA plasmids obviously decreased its expression in BEAS-2B cells upon LPS treatment (Fig. 2A). Through immunoblot assays, we noticed that LPS treatment upregulated the expression of ANXA3 in BEAS-2B cells,



F I G U R E 2. ANXA3 knockdown promoted the survival and suppressed the apoptosis of LPS-induced BEAS-2B cells. (A) Immunoblot showed the expression of ANXA3 in BEAS-2B cells upon the transfection of the indicated plasmids. (B) Immunoblot showed the expression of ANXA3 in BEAS-2B cells upon the indicated treatment. (C) MTT assays showed the survival of BEAS-2B cells upon the indicated treatment. (D) Flow cytometry (FCM) assays were performed and the apoptosis ratio of BEAS-2B cells upon the indicated treatment was shown. (E) Immunoblot showed the expression of Bax, Bcl-2, and cleaved caspase-3 in BEAS-2B cells upon the indicated treatment. Results are presented as mean ± SEM, LPS vs control, ** $p < 0.01$, LPS + shANXA3 vs LPS + shNC, ## $p < 0.01$. NC, negative control.

which was consistent with the previous data, whereas ANXA3 shRNA transfection decreased the expression of ANXA3 upon LPS-treatment (Fig. 2B). MTT assays results showed that LPS-induced BEAS-2B cells showed a decreased survival rate of BEAS-2B cells, whereas ANXA3 depletion rescued the decrease in survival rates caused by LPS treatment (Fig. 2C).

Additionally, we performed FCM assays to detect the effects on cell apoptosis. We found that LPS-induced BEAS-2B cells showed a high apoptosis ratio compared with control cells (Fig. 2D). Notably, ANXA3 ablation reversed the increased apoptosis ratio caused by LPS treatment in BEAS-2B cells (Fig. 2D). Subsequently, we performed immunoblot assays, and found that LPS treatment decreased Bcl-2 expression, and increased Bax and cleaved caspase-3 expression in BEAS-2B cells (Fig. 2E). The depletion of ANXA3 obviously decreased the expression of Bax and cleaved caspase-3 and increased the expression of Bcl-2 upon LPS treatment in BEAS-2B cells (Fig. 2E). Therefore, these data suggested that ANXA3 knockdown promoted the survival and suppressed the apoptosis of LPS-induced BEAS-2B cells.

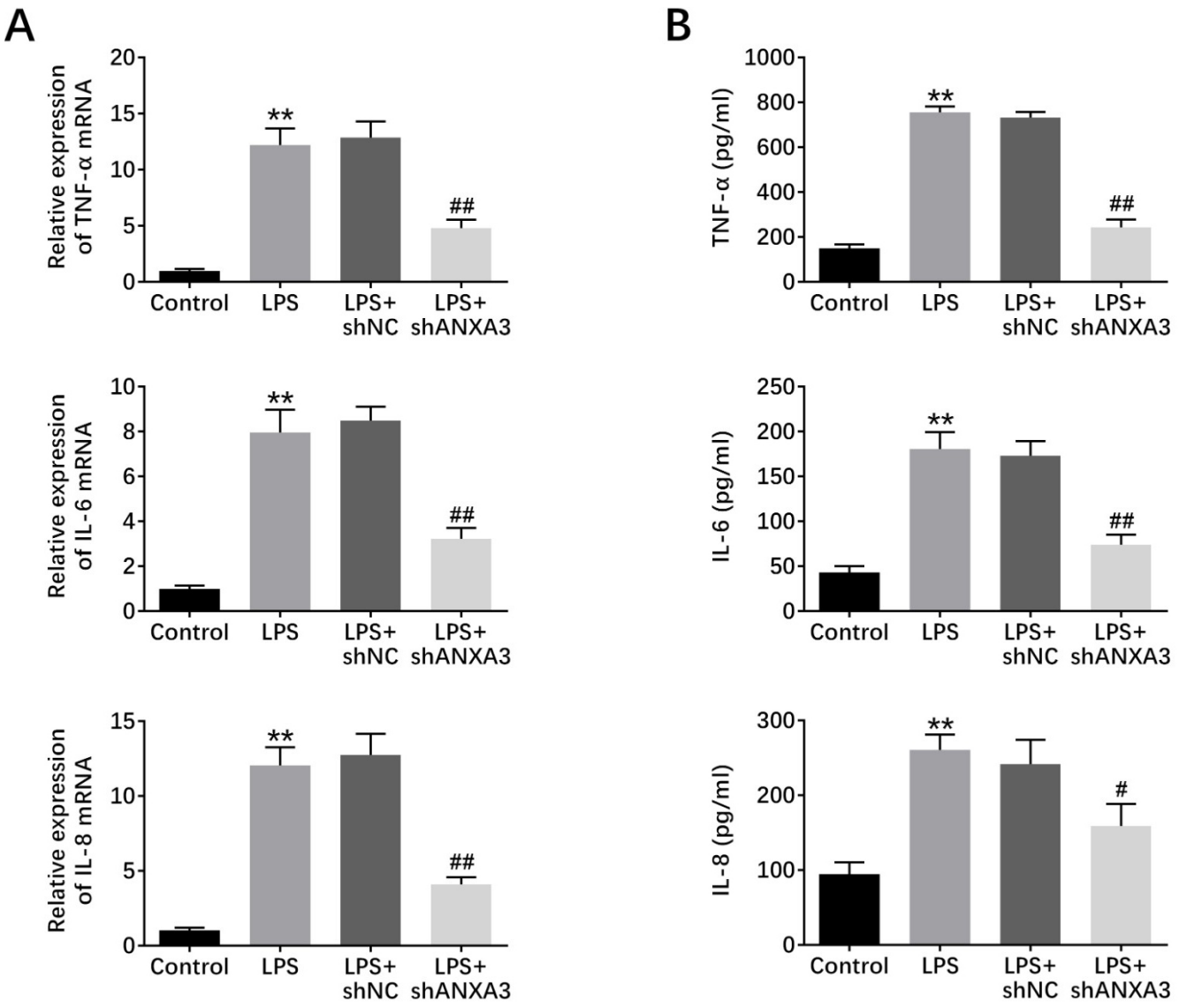


FIGURE 3. ANXA3 knockdown inhibited LPS-induced inflammatory response in BEAS-2B cells. (A) qPCR assays showed the mRNA levels of TNF-α, IL-6, and IL-8 in BEAS-2B cells upon the indicated treatment. (B) ELISA assays showed the levels of TNF-α, IL-6, and IL-8 in BEAS-2B cells upon the indicated treatment. Results are presented as mean $\pm$ SEM, LPS vs control, ** $p < 0.01$, LPS + shANXA3 vs LPS + shNC, # $p < 0.05$, ## $p < 0.01$.

3.3 ANXA3 knockdown inhibited LPS-induced inflammatory response in BEAS-2B cells

Since inflammation was correlated with the progression of asthma, we then explored the effects of ANXA3 on LPS-induced inflammatory response. We assessed the mRNA levels of TNF-α, IL-6, and IL-8 in BEAS-2B cells by qPCR (Fig. 3A). We found that LPS treatment dramatically increased the mRNA levels of TNF-α, IL-6, and IL-8 in BEAS-2B cells, whereas ANXA3 depletion decreased the mRNA levels of TNF-α, IL-6, and IL-8 in BEAS-2B cells upon LPS treatment (Fig. 3A). ELISA assays results showed that TNF-α, IL-6, and IL-8 were all upregulated in LPS-induced BEAS-2B cells, whereas ANXA3 depletion dramatically decreased the levels of TNF-α, IL-6, and IL-8 in LPS-induced BEAS-2B cells (Fig. 3B). Altogether, these results indicated that ANXA3 knockdown inhibited LPS-induced inflammatory response in BEAS-2B cells.

3.4 ANXA3 depletion inhibited HIF1α-induced NLRP3 inflammasome activity

Previous study showed that ANXA3 regulated the activity of HIF1α, and inhibition of HIF1α attenuated inflammatory response in asthmatic mice. We therefore detected the expression of HIF1α through immunoblot assays. We found that LPS treatment significantly increased HIF1α expression in BEAS-2B cells, and ANXA3 depletion obviously decreased the expression of HIF1α in LPS-induced BEAS-2B cells (Fig. 4A).

To examine the effect of HIF1α on NLRP3 inflammasomes, we treated cells with HIF1α inhibitor Echinomycin, and found that Echinomycin significantly inhibited the expression of LPS-induced inflammasome activity indicators, which was similar to the previous references. Several studies also showed that HIF1α promoted the activation of NLRP3 inflammasome, we therefore detected the effects of ANXA3 on the NLRP3 inflammasome in BEAS-2B cells. Immunoblot

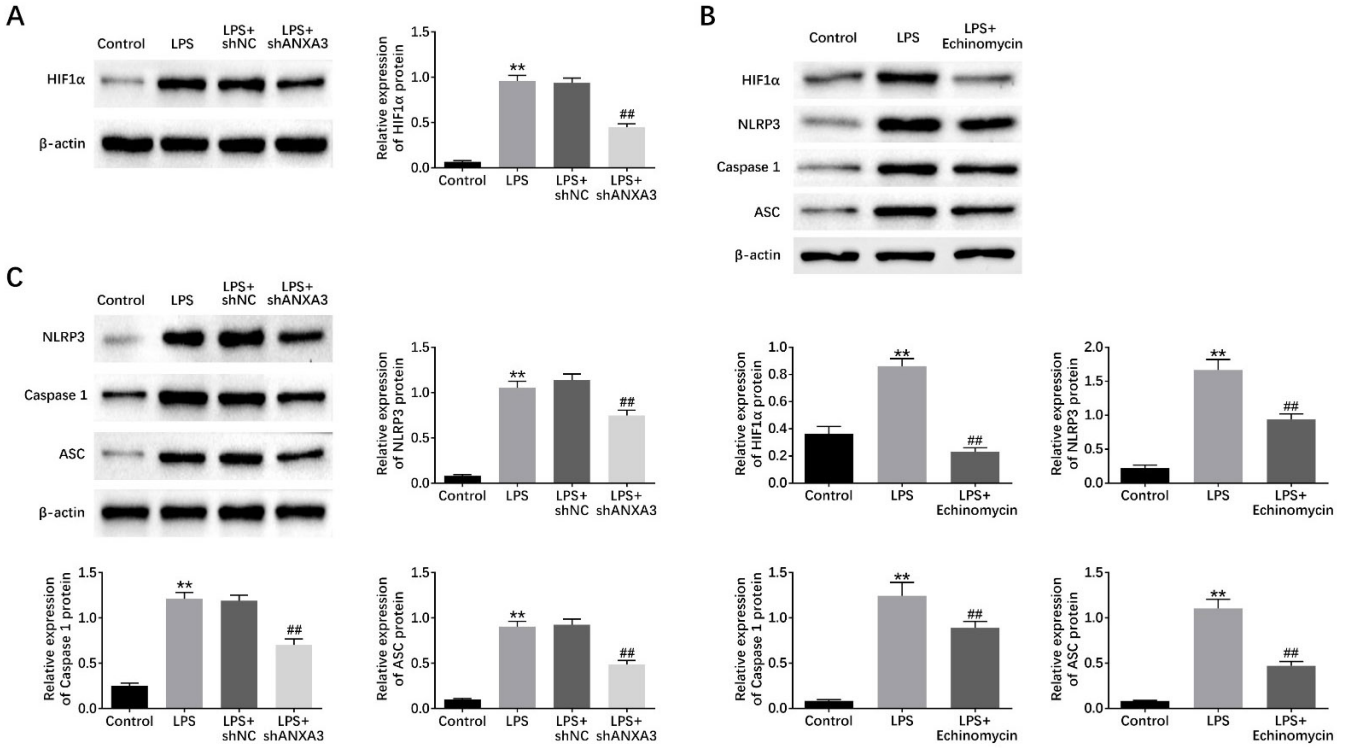


FIGURE 4. ANXA3 depletion inhibited HIF1α-induced NLRP3 inflammasome activity. (A) Immunoblot showed the expression of HIF1α in BEAS-2B cells upon the indicated treatment. (B) Immunoblot showed the expression of NLRP3, Caspase1, and ASC in BEAS-2B cells upon the indicated treatment. (C) Immunoblot showed the expression of NLRP3, Caspase1, and ASC in BEAS-2B cells upon the indicated treatment. Results are presented as mean $\pm$ SEM, LPS vs control, ** $p < 0.01$, LPS + shANXA3 vs LPS + shNC, ## $p < 0.01$.

assays results showed that HIF1alpha inhibitor Echinomycin treatment suppressed the activation of NLRP3 and inhibited the expression of Caspase 1 and ASC in LPS induced BEAS-2B cells (Fig. 4B). Additionally, we found the increased expression levels of NLRP3, and the members of NLRP3 inflammasome, Caspase 1 and ASC in LPS-induced BEAS-2B cells (Fig. 4B). Importantly, the expression of NLRP3, Caspase 1, and ASC was all decreased after ANXA3 depletion in LPS-induced BEAS-2B cells (Fig. 4B). Therefore, ANXA3 depletion inhibited HIF1α-induced NLRP3 inflammasome activation.

4. Discussion

Asthma is related to inflammation. If there is an inflammatory infection, it may trigger asthma [15]. The main characteristic of asthma is chronic airway allergic inflammation caused by bronchial irritants [15]. Asthmatic airway inflammation is mainly featured by cytokine secretion from activated Th2 cells, which directly activates a variety of inflammatory cells such as mast cells, eosinophils and alveolar macrophages, causing them to infiltrate and aggregate in the airway [1]. When the airway is stimulated by allergens or other stimuli, a variety of inflammatory cells release inflammatory mediators and cytokines, the airway epithelium is damaged and the subepithelial nerve endings are exposed, leading to airway hyperresponsiveness [16]. To combat this disease, it is still necessary to further explore the pathogenesis of its inflammatory response and find potential effective therapeutic targets and drugs. In this study, we noticed that a member of the Annexin family, ANXA3, was over-expressed in an inflammatory response model of bronchial epithelial cells. We further confirmed that ANXA3 increased the inflammatory response of LPS-induced bronchial epithelial cells, and thought that ANXA3 could serve as a promising therapeutic target for the treatment of asthma.

The multiple biological functions of ANXA3 have been widely revealed [11, 17]. ANXA3 was involved in the progression of multiple types of tumors [11, 18]. ANXA3 could upregulate the infiltrated neutrophil-lymphocyte ratio, thereby remodeling the immune microenvironment in hepatocellular carcinoma [8]. Another study showed that microglial ANXA3 promoted the development of melanoma via activation of HIF1α/VEGF pathway [12]. Similarly, here, we also found that ANXA3 increased the inflammatory response in an asthma model through HIF1α pathway by the regulation of NLRP3 inflammasome activity [12]. ANXA3 could also negatively modulate lipid storage in clear cell renal cell carcinoma cells and its deletion suppressed the resistance of lung cancer cells to oxaliplatin [19]. Here, we found a novel function of ANXA3 in a nonneoplastic disease, and the precise mechanism needs further study [11].

Several studies showed the effects of ANXA3 on other diseases [20]. ANXA3 was necessary for parallel artery-vein alignment in retina of mice [21]. ANXA3 ablation ameliorated intracranial aneurysm via suppression of JNK pathway [10]. Notably, the data of bioinformation analysis showed the high expression of ANXA3 in asthmatic patients, suggesting that

ANXA3 affected the progression of asthma [22]. ANXA3 promoted the activity of HIF1α, which affected inflammatory response in asthmatic model mice [14]. HIF1α also promoted the activation of NLRP3 inflammasome and increased inflammatory response in bleomycin-induced acute lung injury [7]. Moreover, HIF1α also promoted NLRP3 inflammasome activation in bladder cancer [7]. Therefore, we speculate that ANXA3 promoted NLRP3 inflammasome activation in LPS-induced bronchial epithelial cells. As we expected, we noticed that ANXA3 could increase the inflammatory response of LPS-induced bronchial epithelial cells via mediating HIF1α-induced NLRP3 inflammasome activity, which was consistent with the previous studies.

It was also widely revealed that NLRP3 inflammasome played an important role in asthma [23]. In addition, NLRP3 inflammasome was activated in the bronchial epithelial cells of asthmatic mice and increased the release of inflammatory factors [24]. Here we revealed that ANXA3 promoted HIF1α-induced NLRP3 inflammasome activity. In addition, the correlations between HIF1α pathway and asthma progression have been widely revealed, and our data provided the evidence that HIF1α-induced NLRP3 inflammasome activity was critical in the progression of asthma.

ANXA3 depletion attenuated resistance to oxaliplatin and stimulated apoptosis in colorectal cancer via the MAPK pathway [25]. Additionally, silencing of ANXA3 ameliorated intracranial aneurysm and induced apoptosis through JNK pathway [26]. Here, we found that ANXA3 knockdown promoted the survival and suppressed the apoptosis of LPS-induced BEAS-2B cells, and the possible mechanism needs to be investigated in the next study.

5. Conclusions

In conclusion, in this study, we found that ANXA3 was overexpressed in an inflammatory response model of bronchial epithelial cells and increased the inflammatory response of LPS-induced bronchial epithelial cells via mediating HIF1α-induced NLRP3 inflammasome activity. We therefore thought ANXA3 could serve as a promising therapeutic target for treatment of asthma.

AUTHOR CONTRIBUTIONS

SRZ and QMS designed the study, supervised the data collection. LHJ analyzed the data, interpreted the data. FZ prepare the manuscript for publication and reviewed the draft of the manuscript. All authors have read and approved the manuscript.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AVAILABILITY OF DATA AND MATERIALS

All data generated or analyzed during this study are included in this published article.

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