Original Paper

LPS Exposure in Early Life Protects Against Mucus Hypersecretion in Ovalbumin-Induced Asthma by Down-Regulation of the IL-13 and JAK-STAT6 Pathways

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Key Words

Lps • Asthma • Mucus hypersecretion • IL-13 • JAK-STAT6

Abstract

Background/Aims: Previous studies have shown that lipopolysaccharide (LPS) exposure may have a protective effect on asthma by reducing airway hyper-responsiveness, airway inflammation and serum IgE levels. However, there are few studies investigating the effect of LPS on mucous secretion in asthma. In this study, we evaluate the relationship between LPS pre-treatment in infant mice and airway mucus hypersecretion in an OVA (ovalbumin)-induced asthma model, and further explore the mechanisms behind this effect. **Methods:** Mice were pre-treated with LPS by intranasal instillation (i.n.) from the 3rd day of life for 10 consecutive days before the OVA-induced asthma model was established. In order to detect mucus secretion, periodic acid-Schiff (PAS) staining was carried out, and the expression of Muc5ac was detected. The IL-13 levels in Bronchoalveolar lavage fluid (BALF) and lung tissue were also detected. *In vitro*, the expression of Muc5ac mRNA and protein was quantified in IL-13-stimulated 16HBE cells with or without LPS pre-treatment. In addition, proteins in the JAK2/STAT6 pathway, transcription factors (forkhead box transcription factor A2 (FOXA2), activation protein-1(AP-1), NF-κB), and the levels of reactive oxygen species (ROS) were also measured *in vivo* and *in vitro*. **Results:** LPS pre-treatment reduced mucus secretion, as demonstrated by decreased PAS staining and muc5ac expression. Further exploration of the underlying mechanisms of this phenomenon revealed that LPS pre-treatment decreased the production of IL-13, IL-13 induced ROS synthesis was reduced, and the JAK2/STAT6 pathway

was inhibited. Decreased stat6 increased transcription factor FOXA2, and the relatively increased FOXA2 further decreased the level of Muc5ac and mucous hypersecretion in OVA-induced asthma. **Conclusions:** LPS pre-treatment ameliorated mucus hypersecretion in an OVA-induced asthma model by inhibition of IL-13 production and by further inhibiting the JAK2/STAT6 pathway and ROS activity, and up-regulating expression of FOXA2.

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Introduction

Asthma is a complex inflammatory disease in the lungs characterized by infiltration of the bronchial mucosa by mast cells, lymphocytes and eosinophils, goblet cell hyperplasia, together with activation of T cells. Mucus hypersecretion is an important pathological feature of bronchial asthma, which has become an independent risk factor for the prognosis of asthma. Therefore, it is important to develop drugs that inhibit mucus hypersecretion in susceptible patients. Before addressing these issues, more information is required on mucus physiology and pathophysiology. The mucin family includes Muc2, Muc4, Muc5ac and Muc5b, among others. Among these, Muc5ac is mainly expressed in airway epithelium goblet cells, thus Muc5ac is the most important representative of mucus in asthma [1].

Asthma is associated with Th2 polarization, which produces interleukin-4 (IL-4), IL-5, IL-9 and IL-13 [2]. Among those, IL-13 is now thought to be especially critical. In animal models of allergic asthma, the administration of IL-13 markedly increased mucous hypersecretion [3]. However, after the application of IL-13 monoclonal antibodies or the knockout of IL-13 in asthmatic mice, goblet cell metaplasia in the airways significantly weakened or disappeared [4, 5].

Endotoxin (lipopolysaccharide, LPS), a major component of the outer cell wall of Gram negative bacteria, is ubiquitous in the environment. Epidemiological evidence suggests that exposure to LPS in the environment can protect against asthma by reducing airway hyper-responsiveness, airway inflammation and serum IgE levels through down-regulation of the Th2 response [6-8]. In addition, our previous study has shown that LPS exposure down-regulates the response of Th2 cells, and decreases the levels of cytokines released by Th2 cells. This was especially true for IL-13, which is an important factor to induce mucus hypersecretion (unpublished data). Thus, we investigated whether LPS pre-treatment could decrease mucus hypersecretion and sought to understand the mechanisms underlying this effect.

Materials and Methods

Experimental Animals

BALB/c mice were kept in a specific pathogen free condition and received sterile OVA-free food and water. The mice were maintained on a 12 hour light/dark cycle, under constant room temperature (24°C) and relative humidity (40-80%). The experimental protocols shown in this study were approved by the Institutional Animal Care and Research Advisory Committee at Chongqing Medical University. The use of animals in these experiments was in accordance with the guidelines issued by the Chinese Council on Animal Care.

Neonatal LPS pre-treatment and OVA sensitization and challenge

In a pilot experiment, different doses of LPS were administered at different times to find the optimal dose and "time window" for LPS protection in the OVA-induced asthma model. From the 3rd day of life, mice were exposed to 1 µg LPS (*Escherichia coli* serotype 0111:B4; Sigma-Aldrich) dissolved in 10 µl sterile phosphate buffer (PBS) by intranasal instillation (i.n.) for consecutive 10 days. The control animals received sterile PBS i.n. After LPS exposure was successfully established, the OVA-induced mouse model of asthma was established in the first 6 weeks of life according to previous published methods [9].

Airway hyper-responsiveness (AHR)

AHR was measured 24 hours after ovalbumin challenge by measuring the lung resistance (LR). Animals were anaesthetized with pentobarbital (30 mg/kg ip) and connected via a tracheostomy tube to a computer-controlled small animal ventilator (flexiVent, Scireq). Mice were then challenged with acetyl- β -methacholine (Sigma-Aldrich, Saint Louis, MO, USA), at increasing doses: 0, 3.125, 6.25, 12.5, 25 and 50mg/mL.

Bronchoalveolar lavage and cell counting

Within 24 h after the final challenge, mice were sacrificed and the trachea was cannulated. Bronchoalveolar lavage fluid (BALF) was obtained by flushing the lungs three times with 1.5 mL PBS. Total BALF cell numbers, eosinophils and neutrophils were counted after Wright-Giemsa staining, based on standard morphologic and staining characteristics of 200 cells per sample. The percentage and absolute numbers of each cell type were calculated. The supernatant was stored at -80°C.

BALF cytokine measurements and serum IgE

IL-13 (NeoBioscience, Shenzhen, China) concentrations in BALF were measured with commercial enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's instructions. Serum was separated to assess IgE levels using a murine IgE ELISA kit (NeoBioscience, Shenzhen, China).

Histological analysis

Lung tissues from the mice were harvested and embedded in paraffin. The paraffin blocks were serially sectioned at 4 μ m thickness and stained with hematoxylin-eosin (H&E) and then stained with periodic acid-Schiff (PAS) and Alcian blue (AB). These stains allow the identification of mucus glycoconjugates and mucus-producing goblet cells in accordance with standard protocols. Immunohistochemical (IHC) analysis was performed with antibodies specific to Muc5ac (Abcam, USA). Briefly, after IHC staining, cells stained light yellow to brown were recorded as positive for immunostaining. Images were captured under a Nikon Eclipse E200 microscope connected to a Nikon Coolpix 995 camera (Nikon, Tokyo, Japan).

Normal human bronchial epithelial cell culture

The human bronchial epithelial cell line-16 (16HBE) cells were cultured in RPMI-1640 medium (10% fetal bovine serum and 1% weight per volume penicillin/streptomycin). Cells were maintained in a humidified atmosphere of 5% CO₂ in air at 37°C.

Treatment of 16HBE cells

The 16HBE cells were serum starved for 24 h to maintain a low basal level of Muc5ac production. Three days after the LPS pre-treatment (10 ng/mL), the cells were further treated with or without IL-13 (10 ng/mL) for 12 hours [5]. The negative control group was incubated with PBS only. The cells were divided into four groups: the LPS pre-treatment and IL-13 stimulated (LPS/IL-13); the PBS pre-treatment and IL-13 stimulated (PBS/IL-13); the LPS pre-treatment and PBS stimulated (LPS/PBS); the PBS pre-treatment and PBS stimulated (Control). At the end of stimulation, the cells were collected for further evaluations.

Reactive oxygen species (ROS) assay

For reactive oxygen species generation, the harvested lung homogenates were incubated with 100 μ M 6-carboxy-2',7'-dichlorofluorescein diacetate (DCFH-DA) for 30 min at 37°C. DCFH-DA forms a fluorescent product, DCF (dichlorofluorescein) upon oxidation with ROS. Fluorescence caused by DCF in each well was measured and recorded for 30 min at 485 nm (excitation) and 530 nm (emission) according to the method of Wang and Joseph [10]. The background fluorescence caused by the buffer and by DCF were subtracted from the total fluorescence in each well. Fluorescence intensity was expressed as ROS generation (% control).

Immunofluorescence

16HBE Cells were fixed with formaldehyde before overnight incubation at 4°C with the primary antibody (anti-Muc5ac) diluted 1/100, then the cell were stained with secondary antibody (red). And after washing with PBS, the cell nuclei were stained with DAPI (Blue). Cells were examined using fluorescence confocal microscopy.

Flow cytometric analysis

The lungs were minced and incubated for 20 min at 37°C in 1 mL of sterile PBS containing 0.2% collagenase I (Sigma-Aldrich). Single pulmonary cell suspensions were obtained by forcing tissue through a 70 µm cell filter (Becton, Dickinson and Company, Franklin Lakes, NJ, USA). Erythrocytes were lysed, and the remaining cells were resuspended in RPMI 1640 medium containing 10% fetal bovine serum. A single-cell suspension from the lung (2×10^6 cells/mL) was incubated for 4 - 6 h at 37°C and 5% CO₂ in 1 mL medium containing phorbol 12-myristate 13-acetate (PMA) (50 ng/mL; Sigma-Aldrich), ionomycin (500 ng/mL; Sigma-Aldrich) and GolgiPlug-containing brefeldin A (Becton, Dickinson and Company). The cells were then blocked with rat serum and subsequently stained for surface-associated CD4 (anti-CD4-FITC; Pharmingen of Becton, Dickinson and Company), or CD3 (anti-CD3-PerCP-Cy5.5; Pharmingen). To detect the IL-13 in CD4⁺ T cells in the lungs, the cells were stained for intracellular IL-13 (anti-IL-13-PE-Cy7; Pharmingen), and detected by flow cytometry (FACS Canto; Becton, Dickinson and Company). The data were analyzed with Cell Quest software (Becton, Dickinson and Company).

RNA extraction, reverse transcription and quantitative PCR

The total RNA from mice lung tissues was purified, and cDNA synthesis was performed with a Prime Script RTReagent Kit (Takara, Otsu, Japan). Real-time quantitative PCR (q-PCR) was performed using standard techniques. In each sample, GAPDH was used as an internal control. The primer sequences of IL-13 mRNA were: 5'-CCTGGCTCTTGCTTGCTT-3' (forward)

5'-GGTCTTGTGTGATGTTGCTCA-3' (reverse).

Muc5ac primer sequences were:

5'-CAGCCGAGAGGAGGGTTTGATCT-3' (forward)

5'-AGTCTCTCTCCGCTCCTCTCAAT-3' (reverse).

FOXA2 primer sequences were:

5'-CCCCTGAGTTGGCGGTGGT-3' (forward)

5'-TTGCTCACGGAAGAGTAGCC-3' (reverse).

AP-1 primer sequences were:

5'-AAACGACCTTCTATGACGATGC-3' (forward)

5'-CCGTTGCTGGACTGGATTAT-3' (reverse).

NF-κB primer sequences were:

5'-CAGTGAAGACCCTCTCTCAGG-3' (forward)

5'-AGAGTTAGCAGTGAGGCACCA-3' (reverse).

p-STAT6 primer sequences were:

5'-GCATCTTGCCGCACATCA-3' (forward)

5'-GGTTCGCAGGACTTCATC-3' (reverse).

GAPDH primer sequences were:

5'-AGCAATGCCTCCTGCACCACCAAC-3' (forward)

5'-CCGGAGGGGCCATCCACAGTCT-3' (reverse).

The 16HBE cells were washed twice with PBS and 1 mL of Trizol was added for RNA extraction. The RNA pellet was then dissolved in RNase-free water and stored at -80°C until use. The primer sequences of hMuc5ac were:

5'-TCAACGGAGACTGCGAGTACAC-3' (forward)

5'-TCTTGATGGCCTTGAGCA-3' (reverse).

hp-STAT6 primer sequences were:

5'-AGACCAGGACCTACGGACATACC-3' (forward)

5'-CTCTGCGAAGATACAGCCAACAC-3' (reverse).

hFOXA2 primer sequences were:

5'-CGTCCGACTGGAGCAGCTACTA-3' (forward) and

5'-ATGTACGTGTTTCATGCCGTTCA-3' (reverse).

hAP-1 primer sequences were:

5'-TTCAAATAATCAACTTT-3' (forward)

5'-TAAGTTTATTAGTTGAA-3' (reverse).

hNF-κB primer sequences were:

5'-CAGAGAGGATTTTCGTTCCGTTATG-3' (forward)

5'-GCAGATTTTGACCTGAGGGTAAGAC-3' (reverse).

hGAPDH primer sequences were:

5'-ACTCGTGCTGGGTGGTGGTG-3' (forward)

5'-CTGCAGGCACGTCATGAA TCT-3' (reverse).

Western blot

The total proteins from mice lung tissues and 16HBE cells were isolated, and the protein concentrations were determined using a bicinchoninic acid assay. The proteins from whole-cell lysates were used for Western blot using standard techniques as reported [11]. Anti-Muc5ac (Abcam, USA), anti-JAK2, anti-p-JAK2, anti-STAT6, anti-p-STAT6 (1:2000) (Cell Signaling Technology, USA) antibodies were used. Densitometry of bands from Western blots was done by ImageJ2x 2.1.4.7 (Wayne Rasband, National Institutes of Health, USA), and the density of the Muc5ac, JAK2, p-JAK2, STAT6, p-STAT6 proteins relative to β -actin was measured.

Statistical analyses

The data are expressed as the mean with the standard error of the mean (SEM). All data were analyzed using SPSS 17.0 software. The statistical analyses of LR were performed with two-way ANOVAs and for WB analysis with one-way ANOVAs and the Student's t-test. Differences with P-values less than 0.05 were considered to be statistically significant.

Results

Effect of LPS pre-treatment on an OVA-induced asthma model

The OVA-induced mouse model of asthma was established in the first 6 weeks of life (Fig. 1). To identify the effect of LPS pre-treatment on lung function in an OVA-induced asthma model, AHR of LR was detected by invasive lung function testing. Our OVA-induced asthma model (PBS/OVA) showed an increased AHR compared with control mice challenged with PBS, and LPS pre-treatment (LPS/OVA) ameliorates the AHR compared with PBS/OVA mice (Fig. 2A, $P < 0.05$). Histological evaluation of lung tissue in the PBS/OVA group revealed higher levels of peribronchial inflammation compared with the control group. However, peribronchial inflammation was significantly reduced in LPS/OVA mice compared with PBS/OVA mice (Fig. 2B, $P < 0.05$). Besides, as compared with the control group, mice in the PBS/OVA group showed a significant increase in serum IgE levels and total cell numbers, as well as eosinophils and neutrophils in BALF. However, LPS pre-treatment (LPS/OVA) significantly reduced all these changes compared with the PBS/OVA group (Fig. 2C and 2D, $P < 0.05$).

LPS pre-treatment ameliorates mucus secretion

To determine if LPS pre-treatment could exert protective effects on OVA-induced mucus secretion, lung histologic sections were harvested 24h after the last provocation. The control group showed little mucus production, whereas OVA-challenged mice (PBS/OVA) exhibited goblet-cell hyperplasia and mucous hypersecretion within the airway epithelia. However, LPS pre-treatment significantly reduced OVA-induced mucus secretion in LPS/OVA mice

Fig. 1. Experimental design and protocols employed in this study. From the 3rd day of life BALB/c mice were exposed to 1 μ g LPS i.n. dissolved in 10 μ l PBS or PBS alone for 10 days consecutively, and were sensitized and challenged with or without OVA from the 6th week of life (Grouping: Control, PBS/OVA, LPS/OVA, LPS/PBS group). At day 82, all mice were sacrificed. $n = 6-8$ mice per group.

Fig. 2. Effect of LPS pre-treatment on an OVA-induced asthma model. LPS pre-treatment induced a decrease in AHR, inflammatory peribronchial neutrophils, and serum IgE levels as well as lower total cell count, eosinophils and neutrophils in BALF (Grouping: Control, PBS/OVA, LPS/OVA, LPS/PBS) ($n = 6-8/\text{group}$). **A.** Invasive lung function: AHR in reaction to increased doses of methacholine was measured 24 h after the final challenge. Data are shown as the mean $\pm$ SEM of LR from experimental and control groups of mice. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ as compared with the PBS/OVA group. **B.** HE staining: representative lung tissue sections of each group were stained with hematoxylin and eosin (HE) 24 h after the final challenge. Magnification: $\times 200$ and $\times 400$. **C.** Serum IgE: serum IgE was measured by ELISA. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ as compared with the PBS/OVA group. **D.** Total cell count (a), neutrophils (b), and eosinophils (c) were counted from BALF collected 24 h after the final challenge. Data are means $\pm$ SEM, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ as compared with the PBS/OVA group.

Fig. 3. LPS pre-treatment decreases mucous secretion in an OVA-induced asthma model. PAS-positive intracellular mucin granules populated the epithelium, magnification: $\times 200$ and $\times 400$ (A). Muc5ac protein expression level (B). Quantification of Muc5ac protein expression level (C). Representative images of Muc5ac immunohistochemical staining in lung sections, magnification: $\times 400$ (D). q-PCR of Muc5ac mRNA (E). Data are means $\pm$ SEM, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ as compared with the PBS/OVA group ($n = 6-8/\text{group}$).

compared with PBS/OVA mice (Fig. 3A). As Muc5ac is a representative of airway goblet cell hyperplasia [1], we further measured the expression of Muc5ac. The results demonstrated that the immunohistochemical staining of Muc5ac is higher in OVA-induced asthma (PBS/OVA) compared with control mice, while it is decreased in LPS pre-treated mice (LPS/OVA) compared with PBS/OVA mice (Fig. 3D, $P < 0.05$). In parallel, Muc5ac protein levels and Muc5ac mRNA is elevated in the PBS/OVA group compared with the control group, while LPS pretreatment significantly decreased Muc5ac protein expression and Muc5ac mRNA in the LPS/OVA group compared with the PBS/OVA group (Fig. 3B, 3C and 3E, $P < 0.05$).

Decreased IL-13 levels in LPS pre-treated OVA-induced mice

Since we found that mucus hypersecretion is decreased in LPS/OVA mice, we further studied the mechanisms underlying this finding. It has been reported that the Th2-related cytokine IL-13 was increased in asthma, which could significantly increase mucous hypersecretion [5]. Thus, we measured the IL-13 concentrations in our mouse model. Our results showed that IL-13 expression on $CD4^+$ T cells, IL-13 cytokines in BALF and IL-13 mRNA

in lung tissue were all increased in the PBS/OVA mice compared with the control mice, while they were significantly decreased in the LPS/OVA mice compared with the PBS/OVA mice (Fig. 4, $P < 0.05$). Therefore, our study showed that LPS pre-treatment can down-regulate IL-13 in our OVA-induced asthma model.

Effect of LPS pre-treatment on Muc5ac expression in cultured 16HBE cells

To further investigate the effect of decreased IL-13 on the regulation of Muc5ac, we measured the Muc5ac levels after IL-13 stimulation in 16HBE cells. In an *in vitro* experiment, IL-13 stimulated 16HBE cells induced increased Muc5ac immunofluorescence intensity, Muc5ac protein expression and Muc5ac mRNA in cells given PBS/IL-13 compared with the control cells (Fig. 5, $P < 0.05$). However,

Muc5ac immunofluorescence intensity, Muc5ac protein expression and Muc5ac mRNA were decreased significantly in cells pre-treated with LPS (LPS/IL-13) compared with PBS/IL-13 (Fig. 5, $P < 0.05$). Therefore, we conclude that the decreased IL-13 following LPS pre-treatment could be responsible for the Muc5ac expression *in vivo* and *in vitro*, which may therefore ameliorate mucus hypersecretion in asthma.

Fig. 4. IL-13 production is decreased in LPS pre-treated mice. IL-13 expression as measured by flow cytometry (FCM) (A). Cytokines of IL-13 production in BALF as measured by ELISA (B). Expression of IL-13 mRNA as measured by q-PCR (C). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ as compared with the PBS/OVA group ($n = 6-8/\text{group}$)

Fig. 5. Decreased Muc5ac expression in LPS/IL-13 cells compared with PBS/IL-13 cells. Grouping: LPS pre-treatment and IL-13 stimulated (LPS/IL-13); PBS pre-treatment and IL-13 stimulated (PBS/IL-13); LPS pre-treatment and PBS stimulated (LPS/PBS); PBS pre-treatment and PBS stimulated (Control). Confocal microscopy of Muc5ac immunofluorescence, magnification: $\times 400$ (A). Western blot of Muc5ac protein levels (B). Quantification of Muc5ac protein levels (C). q-PCR of Muc5ac mRNA (D). Data are means $\pm$ SEM, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ as compared with the PBS/IL-13 group, ($n = 6-8/\text{group}$).

Effect of LPS pre-treatment on p-JAK2, p-STAT6 and ROS generation in mice and 16HBE cells

We further studied the specific mechanisms of how a decrease in IL-13 stimulated by LPS pre-treatment could result in decreased Muc5ac levels. The mechanism by which IL-13 regulates mucin synthesis is complicated, including mitogen-activated protein kinase/

Fig. 6. Decreased expression of p-JAK2, p-STAT6 and reactive ROS, and increased FOXA2 mRNA in LPS pre-treated mice. Western blots of t-JAK2, t-STAT6, p-JAK2 and p-STAT6 protein expression in lung tissues (A). Expression of p-STAT6 mRNA in lung tissues as measured by q-PCR (B). Reactive ROS in lung homogenate (C). Expression of FOXA2, AP-1 and NF- κ B mRNA in lung tissues as measured by q-PCR (D). Data are means $\pm$ SEM, * P <0.05, ** P <0.01, *** P <0.001 as compared with the PBS/OVA mice (n = 6–8/group).

Fig. 7. Decreased expression of p-JAK2, p-STAT6 and reactive ROS, and increased FOXA2 mRNA in LPS pre-treated 16HBE cells. Western blotting of t-JAK2, t-STAT6, p-JAK2 and p-STAT6 protein expression in 16HBE cells (A). Expression of p-STAT6 mRNA in 16HBE cells as measured by q-PCR (B). Reactive ROS in 16HBE cells (C). Expression of FOXA2, AP-1 and NF- κ B mRNA in 16HBE cells as measured by q-PCR (D). Data are means $\pm$ SEM, * P <0.05, ** P <0.01, *** P <0.001 as compared with the PBS/IL-13 cells.

extracellular regulated protein kinases pathways (MAPK/ERK), Janus Kinase-Signal Transient and Activator of Transcription pathways (JAK-STAT) and the p38/MAPK pathway [12]. Therefore, western blotting analysis *in vivo* and *in vitro* were performed to investigate these signaling pathways. We detected that p-JAK2 and p-STAT6 were increased in OVA-induced mice (PBS/OVA) and IL-13 stimulated 16HBE cells (PBS/IL-13) compared with the control. However, LPS pre-treatment (LPS/OVA or LPS/IL-13) could significantly inhibit the IL-13-mediated p-JAK2 and p-STAT6 pathway (Fig. 6 A-B and Fig. 7 A-B, P <0.05) *in vivo* and *in vitro*.

In addition, ROS has been implicated in mucous hypersecretion in asthma and has been reported to be activated by IL-13 [13–15]. We also examined the level of ROS *in vivo* and *in vitro*. As shown in Fig. 6C and Fig. 7C, the generation of endogenous ROS was significantly elevated in the PBS/OVA mice and PBS/IL-13 cells compared with the control. After pre-treatment with LPS (LPS/OVA or LPS/IL-13), ROS generation was significantly suppressed (Fig. 6C and Fig. 7C, P <0.05). Thus, reactive ROS in the OVA-induced asthma model was decreased following LPS pre-treatment, and this may be responsible for the decreased IL-13 production. This suggests that IL-13-induced oxidative stress may be ameliorated by LPS pre-treatment.

Effect of LPS pre-treatment on the level of transcription factors

To further detect the transcription factors involved in the expression of Muc5ac, we measured the *in vivo* and *in vitro* levels of FOXA2 mRNA, AP-1 mRNA, and NF- κ B mRNA by q-PCR. We found that there was a significant decrease of FOXA2 mRNA (Fig. 6D and Fig. 7D, P <0.05) in PBS/OVA mice and in PBS/IL-13 16HBE cells compared with the control group. LPS pre-treatment (LPS/OVA and LPS/IL-13) considerably increased the level of FOXA2 mRNA compared with the PBS/OVA mice and the PBS/IL-13 16HBE cells (Fig. 6D and Fig. 7D, P <0.05). However, there was no difference in AP-1 mRNA and NF- κ B mRNA expression *in vivo* and *in vitro*. Therefore, FOXA2 expression, a transcriptional repressor of Muc5ac, was greatly increased after LPS pre-treatment and further down-regulated the Muc5ac gene transcription, thus reducing mucus production.

Discussion

Mucous cell hyperplasia can be observed in the airways of almost every asthma patient and airway mucus plugging can affect the progression or aggravation in asthma or even lead to death [16]. However, there is no good treatment for mucus hypersecretion in asthma, thus specific drugs is required. Therefore, the mechanism of mucous hypersecretion needs to be explored. Inflammatory mediators produced in asthmatic airways can increase mucin secretion, induce plasma exudation, up-regulate muc5ac gene expression (muc5ac gene expression is thought to be a marker of goblet cell hyperplasia) [17], and cause goblet cell hyperplasia [18, 19]. It's worth noting that IL-13, a cytokine released from Th2, is a major driver of asthma in a large subset of individuals, and appears to have a prominent role in MUC5AC expression and mucus production [3, 4, 20-22]. It has been reported that LPS exposure can down-regulate Th2 responses and thereby reduce cytokine IL-13 [8, 23]. Thus, in this study, we explored whether LPS pre-treatment could inhibit mucus hypersecretion and if so, what the underlying mechanisms of this effect were.

In our study, after pre-treatment with LPS before asthma was induced with OVA, the AHR, the peribronchial inflammation, the levels of IgE and IL-13, the goblet cell metaplasia as stained by PAS staining and the expression of Muc5ac in mice were significantly decreased, suggesting that LPS pre-treatment improved asthma by ameliorating AHR, peribronchial inflammation and mucous hypersecretion. *In vitro*, we used LPS pretreatment of IL-13-stimulated 16HBE cells. The results of immunofluorescence and western blotting showed a significant decrease in Muc5ac in the cells. Therefore, our study demonstrated that pre-treatment with LPS inhibited the IL-13-stimulated Muc5ac expression both *in vivo* and *in vitro*.

The JAK/STAT signaling pathway consists of the tyrosine kinase JAK family and the transcription factor STAT family, and is an important cytokine signal transduction pathway [24, 25]. JAK is activated when IL-13 binds to the receptor IL-4R α or the IL-13R α 1 chain, thereby activating the phosphorylation of STAT6 in the cytoplasm. STAT6 is a target protein downstream of the JAKs [24]. Studies have shown that the inflammatory mediators (especially IL-13) synthesized by Th2 cells are upstream stimulators for JAK/STAT6 pathway activation and can induce phosphorylation of STAT6. p-STAT6 is involved in goblet cell metaplasia and leads to excessive mucus secretion [26]. Therefore, the JAK/STAT6 pathway may play an important role in IL-13-induced airway hyper-reactivity and mucus over-production [27]. Thus, we studied the JAK/STAT6 signaling pathway. In our study, we found a significant increase of p-JAK expression in our OVA-induced asthma model, and LPS pre-treatment significantly decreased the levels of p-JAK. As such, we measured the change in STAT6 expression in each group. We found that p-STAT6 expression was significantly increased after OVA or IL-13 stimulation, and LPS pre-treatment inhibited the expression of p-STAT6. In addition, ROS plays an important role in mucus hypersecretion and airway inflammation, and ROS can activate the transcription factors which are sensitive to redox reactions [1, 28]. In addition, ROS is activated by IL-13, which in turn activates downstream signaling pathways. ROS not only activates STAT6 signaling pathways, but also activates STAT6 to stimulate more ROS production to form a positive feedback loop, resulting in substantial mucus secretion [13-15]. Our study also found that ROS levels were significantly increased with the activation of the STAT6 pathway, whereas LPS pretreatment inhibited the pathway activation and decreased ROS levels. The Muc5ac promoter contains FOXA2, AP-1, NF- κ B and other transcription factor binding sites [29-31]. FOXA2 is a transcriptional repressor of Muc5ac and inhibits mucus hypersecretion [32], while AP-1 and NF- κ B can promote transcription of the Muc5ac gene and increase hyperplasia in goblet cells [33-37]. Our results suggest that IL-13 can induce the expression of muc5ac expression, suggesting that FOXA2, AP-1 or NF- κ B plays a role in muc5ac expression. The results of our q-PCR analysis showed that AP-1 and NF- κ B expression did not significantly change after IL-13 stimulation, whereas FOXA2 was decreased in our OVA-induced asthma model, and LPS pre-treatment significantly increased the levels. We hypothesized that increased IL-13 may stimulate the

transcription and protein synthesis of MUC5AC by down-regulating FOXA2 expression, whereas LPS is effective in improving this process.

Conclusion

In summary, we are exposed to various concentrations of LPS in our daily life [38, 39]. Our results show that LPS pre-treatment can suppress IL-13-stimulated ROS generation and the JAK2/STAT6 pathway activation, and further promote transcription factor FOXA2, thus inhibiting Muc5ac expression and mucous hypersecretion in asthma (Fig. 8). Therefore, our results, combined with high-quality epidemiological finding, suggest that LPS pre-exposure in early life could reduce mucous hypersecretion, airway hyper-responsiveness and inflammation, and could thus protect against asthma [6-8]. We speculate that in infants and young children, especially those with a family history of allergy, deliberate exposure to the endotoxin in the environment could reduce the risk of severe asthma. Furthermore, the above information could also be used to create therapeutic targets which, in turn, should lead to the rational design of anti-hypersecretory drugs for treatment of airway mucus hypersecretion in asthma. Thus, our study provides a basis for the creation of treatments for the prevention and treatment of asthma.

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Disclosure Statement

The authors have no conflicts of interest to declare.

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Fig. 8. Signaling pathways involved in IL-13-mediated mucus synthesis. Binding of IL-13 with IL-4Rα or IL-13Rα activates cytokine receptor-associated p-JAK2, resulting in increased p-STAT6. In addition, the increased ROS stimulated by IL-13 can also increase p-STAT6. Following dimerization, p-STAT6 translocates into the nucleus, and suppresses expression of FOXA2, a transcriptional repressor of Muc5ac. Together, these intracellular signaling events illustrate how IL-13 increases Muc5ac expression.

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